Three-dimensional molecular reconstruction of rat heart with mass spectrometry imaging

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ABSTRACT

Cardiovascular diseases are the world's number one death cause, accounting for 17.1 million deaths a year. New high-resolution molecular and structural imaging strategies are needed to understand underlying patho-physiological mechanism. The aim of our study is (1) to provide a molecular basis of the heart animal model through the local identification of biomolecules by Mass Spectrometry Imaging (MSI) (3D molecular reconstruction), (2) to perform a cross species validation of SIMS based cardiovascular molecular imaging, and (3) to demonstrate potential clinical relevance by the application of this innovative methodology to human heart specimens. We investigated a MSI approach using secondary ion mass spectrometry (SIMS) on the major areas of a rat and mouse heart: the pericardium, the myocardium, the endocardium, valves and the great vessels. While several structures of the heart can be observed in individual 2D sections analysed by Meta-SIMS imaging, a full view of these structures in the total heart volume can be achieved only through the construction of the 3D heart model. The images of 3D reconstruction of the rat heart show a highly complementary localization between Na^+, K^+, and two ions at *m/z* 145 and 667. Principal component analysis of the MS imaging data clearly identified different morphology of the heart by their distinct correlated molecular signatures. The results reported here represent the first 3D molecular reconstruction of rat heart by SIMS imaging.

Key words: 3D molecular reconstruction, Secondary Ion Mass Spectrometry, heart, imaging

Non-standard Abbreviations and Acronyms.

SIMS	Secondary Ion Mass Spectrometry
MS	Mass Spectrometry
MSI	Mass Spectrometry Imaging
ToF	Time-of-Flight
MetA-SIMS	Metal Assisted-Secondary Ion Mass Spectrometry
PCA	Principal Component Analysis
VARIMAX	VARIance MAXimization
DAG	DiAcylGlycerols
TAG	TriAcylGlycerols
FFA	Free Fatty Acids
MALDI-MSI	Matrix-Assisted Laser Desorption/Ionization-Mass Spectrometry Imaging

Introduction

Heart Failure is among the leading causes of morbidity and mortality and can result from either primary or secondary heart muscle disease [1]. The causes of cardiac dysfunction in most heart diseases are still largely unknown, but are likely to result from underlying alterations in gene and protein expression or downstream metabolic processes. The functional complexity of an organism far exceeds that indicated by its genome sequence alone and this is dependent on the products of gene expression, including transcriptomics, proteomics, lipidomics and metabolomics [2-5]. Proteome databases containing two-dimensional (2-D) gel electrophoresis, 2-D gel images and protein spot identifications have been compiled for canine and rat myocardial tissues [6,7]. An animal model is crucial to evaluate new basic molecular insights prior to their application in human studies. Rats exhibit physiological characteristics similar to those of humans and have been a key experimental model in biomedicine for over a century [8]. However, to date, there is no comprehensive molecular image database for the rat heart. The construction of such databases in animal model is important for the identification of the molecular basis of pathological substrates caused by a cardiovascular disease. This requires a molecular imaging method that provides detailed insight into the spatial distribution of a broad range of elements and molecules. A mass spectrometer is described as the smallest weighing scale ever used in the world [9]. Mass spectrometry (MS) is an analytical technique that is used to determine the molecular weight of a variety of chemical compounds. Because the mass of a chemical compound is dependent on its elemental composition, it is an important determinant of its identity. Mass spectrometry imaging (MSI) is a technique that enables the identification and localization of molecules directly from biological surfaces. The advantage of MSI is its ability to detect the distribution of hundreds of unknown compounds in a single measurement without the use of chemical or immunological labels [10,11]: it is a true label-free molecular imaging technique. The aims of the present study were: a) the establishment of a molecular atlas of the heart through a direct and large-scale, local analysis of lipid and elemental ions in a healthy rat heart tissue; b) to perform a cross species validation

through the analysis of mouse and human heart tissue and compare and contrast our molecular findings; c) to test this methodology on clinical human samples as the last element of our translational research.

Ethics statement

The animals used in this study were purchased from Harlan Laboratories (Boxmeer, The Netherlands). The Center for Cardiovascular Pathology of the University of Padova specifically approved the use of human tissues. Written informed consent was acquired from human subject involved in the study.

Methods and materials

Rat heart from adult rats (type WU) and mouse heart (type 9CFW-1) were frozen and stored at -80°C until sectioning. The tissue sections were stained with hematoxylin (Sigma Diagnostics, Zwijndrecht, the Netherlands) and eosin (Merck Diagnostica, Darmstadt, Germany) after SIMS analysis to correlate the observed molecular profiles with morphological features. Several successive heart sections from three different rats and one mouse heart were analyzed using standardized and optimized workflows.

Rat and mouse hearts were sectioned at 12 μm thickness and sliced into sagittal sections at −20°C on a cryomicrotome (Microm International, Walldorf, Germany) and then mounted on indium-tin-oxide coated glass slides (ITO, 4–8 Ω resistance; Delta Technologies, Stillwater, MN, USA). All samples were stored at −80°C prior to use and dried in a vacuum desiccator for 30 min prior to MS analysis. The 12 μm thickness ensures that enough analyte molecules are available for ionization and no problems occur with the insulating properties of tissue [12]. Sample metallization as a means

to improve molecular ion yields was performed by sputter deposition of a 1 nm layer of gold [13]. Samples were analyzed by high resolution time-of-flight secondary ion mass spectrometry (ToF-SIMS) imaging. This procedure is often referred to as MetA-SIMS when sample metallization is used. ToF-SIMS analysis of the cross-sectioned heart was done on a Physical Electronics (Eden Prairie, MN) TRIFT-II (triple focusing time-of-flight) ToF-SIMS system, equipped with a 22 keV gold liquid metal ion source. The analysis was performed in positive ion mode. The instrument was calibrated in positive ion mode on high occurrence elements and fragments such as H^+, $C_nH_m^+$, Na^+, K^+. Since the sample size is approximately 2x2 cm, the resulting image fidelity of such ToF-SIMS experiments is close to 0.6 µm per pixel. The raster size (or field of view, FOV) used was 150 µm per tile, with resolution of 256x256 pixel per tile. The FOV is automatically calculated by WinCadence 4.4.0.17 software (ULVAC-PHI Inc., Kanagawa, Japan) based on the maximum raster size defined by the user and the total measurement area. The heart tissue was analysed using a mosaic mode of 128x128 tiles with a spectral resolution of 22 bit mass channels, for a total of about one billion pixels. The acquisition time was three seconds for each tile. Both FOV and acquisition time were constant throughout the experiment, which covered the entire sample surface. SIMS is an extremely surface sensitive technique in which ions are exclusively generated from a depth of no more than 50 nm from the tissue surface. We used the static SIMS mode, where the primary ion dose is so low that each incoming ion hits a unique spot on the surface, less than 1 percent of the surface area is analysed [14,15]. In our work a list is created from the recorded data containing the position as a two-dimensional coordinate, the channel number, *c* (which is linearly related to the time-of-flight and hence the m/z value), and the number of counts (*n*) for that respective ion. This dataset, which represents a so-called datacube, can subsequently be converted into an $x \times y$ by c unfolded datacube containing the number of counts for each spectral and spatial combination. These datacubes can be visualized with the DataCube Explorer (http://www.maldi-msi.org) developed at the FOM Institute AMOLF, that is a lightweight visualization tool providing a platform to share and explore MSI data sets. Several useful features are available to dynamically scroll through the data,

analyze selected regions and process and classify the image [16]. Here we will focus on a description of the high resolution Meta-SIMS molecular atlas that was recently completed from us.

PCA analysis and variance maximization (VARIMAX) rotation

Principal component analysis (PCA) was employed to extract meaningful information out of the complex and extremely large datasets generated by high resolution imaging mass spectrometry (IMS). PCA is a statistical technique to find patterns in data of high dimensionality. The data are described in such a way that correlated similarities and differences are highlighted. This is realized by transforming a number of data features (variables) into a smaller number of orthogonal variables called principal components (PC), which de-facto consist of correlated spectral features. The first principal component accounts for the largest amount of variance in the data. Each successive component describes a smaller part of the remaining variance. PCA is therefore well-suited to be applied on hyperspectral datasets as used to construct the molecular atlas described in this paper. The hyperspectral SIMS data have both a large spatial as well as a large spectral dimension. Additional optimizations can be done after completion of the PCA to enhance the spectral contrast in the data. One method is an additional fitting of the principal components to maximize the variance expressed in each component. There is a number of maximization criteria but the VARIance MAXimization (VARIMAX) is the most common. It can be used as a post-processing step after PCA, as previously described [17]. By rotation of the orthogonal axis, components with a higher contrast are created. To highlight the spectral correlations in the 3-D atlas, PCA was used with VARIMAX optimization.

Rat Heart High-Resolution Imaging Mass Spectrometry Data

Imaging mass spectrometry data is often displayed as a total-ion-count image; the output of a mass spectrometer consists of a set of mass-to-charge ratios (m/z) of detected ions. IMS produces such a set of mass-to-charge ratios for each pixel in a 2D grid, allowing to analyse the chemical structure of the sample. The resulting grid of m/z histograms is commonly visualized in the form of a total-ion-count image. For each pixel, a total-ion-count image maps the number of items in the corresponding set of m/z values to an intensity value. Other methods of visualization are based on various forms of multi-variate analysis (MVA) on the m/z data, producing maps of chemically similar regions as show from Smit et al. [18].

Human heart Human failing left-ventricular free wall heart explants were obtained from the heart transplant collection at Padua University. Sample (left ventricle) from explanted heart (heart failure) were frozen and stored at -80°C until sectioning. Heart tissue transverse sections (12 µm thickness) were cut using a cryomicrotome and thaw mounted onto an ITO slide. The same SIMS-ToF method and statistical analysis described for rat and mouse heart were applied to the human sample. A sample of the left ventricle was further divided into manageable blocks for paraffin embedded. From each block three serial sections of 4 µm thickness were taken. Adjacent (serial) sections were then stained with one of the three following techniques to correlate heart's molecular profile with morphological features: a) Hematoxylin and Eosin (H&E) staining, b) Sirius red-collagen-staining for connective tissue, c) Heidenhain's Azan stained for connective tissue. Comparison was made with a serial staining section to show the anatomical structure and confirm the distribution of connective tissue in that location.

Results

2D MSI based molecular imaging of rat heart sections

Surface rastering of heart tissue sections generated a plethora of secondary ions with a molecular weight up to m/z 1500 (Figure 1 A,B,C). Several distinctive MS peaks and correlated image patterns were observed in the positive-ion mode for the heart sections analyzed. The selected ion images are highly sensitive to the specific anatomical tissue types within the sections (Figure A1,B1). Several peaks are often visible within a single m/z range and a single peak can be used to create descriptive molecular ion images. The resulting intensity map is usually visualized using a pseudo-color map. Several molecular patterns, which are entirely based on the local elemental and lipid composition in accordance with scientific literature [19-22], are clearly distinctive for different structures of the heart. The high spatial resolution images shown in Figure 2 demonstrate that the spatial resolution obtained in these SIMS experiments is more than sufficient to reveal in detail all major anatomical substructures in the rat heart. Note that conventional MALDI-MSI with a pixel size of 100 μm is not capable of revealing the detailed structures shown in Figure 3 where the distribution of a wide variety of secondary ions imaged in the heart is observed. The ions at m/z 667, and m/z 840 localized very precisely within the aorta wall (Figure 3c, 3d). For instance, m/z 83 is localized in aorta wall, semilunar valve and endocardium, whereas it is hardly visible in both ventricles and atrias (Figure 3a). The image f shows the ion at 145 m/z very localized in pulmonary artery, right atria and atrioventricular valve. Interestingly, m/z 175 and m/z 213 are observed only in ventricles. The bottom image m, in contrast, shows m/z 334 localized only in the pericardium The image q shows the high spatial distribution of at 969 m/z in both atrias, aorta wall, left atrioventricular valve and right coronary artery. Significant peaks in our data are seen at m/z 369 ($C_{27}H_{45}$), representing cholesterol ion $[M-H_2O+H]^+$ whose distribution was imaged. Important

tissue differences are distinguished upon examination of the cholesterol distribution. Cholesterol shows higher intensity in both atria, aorta wall, atrioventricular valves and the coronary artery but is observed with lower intensity in ventricles (Figure 3, 3i). VARIMAX rotation was used to enhance the spectral contrast of the PCs. This axis rotation results in a higher molecular contrast not only in the spectra, but also in a higher molecular image contrast. Several signals of fatty acids show a variation in their spatial distribution that corresponds directly to the degree of lipid unsaturation, and hence energy catabolism. DAG species, that were identified as $[M+H-OH]^+$ ($C_{35}H_{65}O_4$) at m/z 549, $[M+H-OH]^+$ ($C_{37}H_{69}O_4$) at m/z 577, $[M+H-OH]^+$ ($C_{39}H_{71}O_4$) at m/z 603 and ceramide at m/z 604 could be detected. Substantial differences are also seen in the amount of free choline present in the various tissue parts. The distributions based on the PCA results revealed a clear image of the different areas where m/z 104 (choline) displayed a high intensity signal. The m/z 104 signal strongly localizes in atrias, aorta, pulmonary artery, atrioventricular and semilunar valves but has lower intensity in ventricles, as shown in Figure 4B. The phosphocholine headgroup at m/z 184 ($C_5H_{15}PNO_4$) was used to localize the phosphocholine-containing phospholipids, i.e. sphingomyelins and phosphatidylcholines [23]. The phosphocholine headgroup was additionally localized by imaging a specific fragment (m/z 86). This signal was localized in the left and right ventricle with low intensity. In right and left atria, aortic wall and aorta valve the intensity of m/z 86 signal was higher (Figure 3a). The localization of chemical components in the tissue reveals structural information that can be used for creating an atlas (Figure 3). Separated ion images of relevant molecules provide this image information. PCA is used to find spatially correlated molecules, the resulting PCA score images greatly enhance image contrast in comparison with the separate ion images (Figure 4).

3-D molecular reconstruction of the rat heart

The generation of a 3-dimensional dataset requires an additional z-dimension. In our experiments this was achieved by successive tissue sectioning with well defined and measured spatial intervals [24]. Using the micrometer scale of a cryomicrotome, an entire cryo-preserved rat heart was sectioned. Forty sections at irregularly spaced, but well documented intervals were taken through the heart as shown in Figure 1(A) (supplement online). The workflow of the 3-D recontruction is show in Figure 5. The z-position was recorded for each of the sections. High resolution Meta-SIMS datasets were acquired from each section with a 22 keV Au^+ primary ion beam. Each dataset was acquired in 12 hours. Forty datasets were acquired resulting in a total of 42,949,672,960 spectra in the raw data files. These data were subsequently combined and processed to reveal the 3-dimensional molecular features. The processing protocol included spectral and spatial binning to reduce the total dataset size prior to molecular feature visualisation using our datacube explorer. The 3-D data volumes can be explored using the Volume explorer software that is used to reconstruct a 3D-data grid out of the 40 individual 2D-datasets. The molecular images of a specific m/z range of interest areas are put together into a three dimensional volume in which the pixels are turned into voxels. The Volume explorer then uses volume-extraction by combining voxels above a certain threshold into a volume. The co-registration of individual molecular images was obtained by manual alignment in the Volume explorer of MSI-section on the base of anatomical structures. This is, to the knowledge of the authors, the first time three dimensional SIMS based molecular volumes were constructed with a 1 µm lateral resolution and a ~100 µm depth resolution. The consistent observation of identical correlated anatomical and molecular structures within each of the technical replicates is show in Figure 1 B supplement online.

The images of the 3D reconstruction additionally show a highly complementary localization between Na^+, K^+, and an ion at m/z 145. Na^+ is localized in the atria, while K^+ is strongly localized in the ventricles as previously reported [25] (Figure 5D). Three dimensional reconstructions of three

selected individual mass spectral peaks can be simultaneously displayed using a Red-Green-Blue (RGB) color scheme. Each color represents a specific molecular feature (Figure 5 E). The overlaid 3D molecular model obtained is a representation of the whole heart. The result of the combination and co-registration of forty individual MS imaging data cubes is demonstrated in Figure 5F. The data volumes can be explored using a software which allows a close molecular look inside the heart. The molecular visualisation of the different valvular structures, the pericardium, the atria, the coronary arteries and various other anatomical features provides a new tool in molecular pathology. While several structures of the heart can be observed in individual 2D sections analysed by Meta-SIMS imaging, a full view of these structures in the total heart volume can be achieved only through the construction of the 3D heart model. The data shows significant differences in the ions distribution in the various heart structures and reveals distinctive molecular localization in this organ.

Mouse heart

A cross-species validation was performed through the analysis of mouse heart sections (Figure 1D,D1,D2). We observed similar molecular patterns when the mouse results were compared to the rat results. This demonstrates among others the diversity in biological systems in which this technology can be applied. Cholesterol related ions again demonstrated to have specific higher intensity in atria, aorta, pulmonary artery and lower intensity in ventricles (Figure 1 D). The ion at m/z 369 localized very precisely within the pulmonary artery, tricuspid with high intensity in both atria, aorta, pulmonary artery, in the atrioventricular and semilunar valves but is detected in ventricles with lower intensity. The similarity of these molecular findings with the results obtained in the rat heart further corroborates the across species molecular consistency at the lipid level.

Human heart

The true relevance of a new molecular imaging technology as described here is demonstrated through the application of high resolution imaging MS on explanted human heart samples taken from patients suffering from ventricular failure. The application of MSI in the field of human cardiovascular pathology has a direct potential for clinical diagnoses and treatments. The larger size of the human heart combined with the time consuming experimental procedure is prohibitively limitative for the generation of a full three dimensional molecular model of the human heart. Instead, we have opted for the analysis of smaller subsets of the human heart, in this case segments of the left ventricle.

In all the experiments the several distinctive structures in the ventricle can be clearly seen, originating from different lipid composition and signal intensities. An abundant signal of cholesterol (m/z 369) $[M\text{-}H_2O+H]^+$ can be observed (Figure 6). Cholesterol ion exhibit a high intensity in the myocardium. The observed intensities in the pericardium and endocardium are substantially lower. The m/z 104 choline signal co-localizes with high intensity in myocardium and endocardium (Figure 6). The oleic (47%) and palmitic (19%) acids are known to be the dominant fatty acids in the human heart. Results from human and animal models of heart failure generally support the concept of decreased fatty acid β-oxidation in heart failure [26]. These findings are consistent with our molecular images presented below that show DAG species tentatively identified as $[M+H\text{-}OH]^+$ ($C_{35}H_{65}O_4$) at m/z 549, $[M+H\text{-}OH]^+$ ($C_{37}H_{69}O_4$) at m/z 577, $[M+H\text{-}OH]^+$ and ceramide at m/z 604. As expected, they could be detected with high intensity in the pericardium and low intensity in the myocardium. The SIMS imaging data demonstrated that several important catabolic molecules and their spatial features can be readily identified and localized in a single MS

imaging experiment (Figure 6). This in turn leads to a better fundamental understanding of the pathological pathways whose molecular constituents are visualized.

Adjacent human left ventricle sections were stained with H&E, Sirius red and trichrome, and compared to the SIMS imaging data sets obtained (Figure 6 A,B,C,D). Figure 6 (B, C and D) illustrates a transverse-section of left ventricular muscle, that contains a vein structure. The different tissue types in this section can only be distinguished by a combination of three different staining procedures, revealing the morphology and the presence of connective tissue. The molecular images obtained with SIMS provide all of this information (and more) in a single experiment. Statistical analysis of human heart (3 technical replicates) yields correlation coefficients of overall spectra of A *vs* B 0.9934, A *vs* C 0.9858, B *vs* C 0.9925, respectively. This again confirms the reproducibility and selectivity of this innovative method for molecular histology (see figure 2 online support). The tissue sections were stained with H&E after SIMS analysis to correlate the heart's molecular profile with the observed morphological features.

Discussion

SIMS imaging was able to consistently detect sodium (23 m/z), potassium (39 m/z), choline (104 m/z), phosphocholine (184 m/z), cholesterol (369 and 385 m/z), DAG species (549 m/z, 577 m/z, 603 m/z), ceramide (604 m/z), the ions at 145 m/z, 175 m/z, 334 m/z, 213m/z, 969 m/z and several other molecules in rat, mouse, and human heart structures. The assignments of molecules were made based on the unique masses of single elements (e.g. Na^+ m/z 22.989, K^+ m/z 39.098), the calculated molecular weight of more complex substances, and by comparison with chemical

standards. The lipid database was employed to correlate specific molecules found by SIMS imaging within the heart structures. The ability to identify specific biomolecules is crucial for biological, and especially pathological, investigations. Molecular biology thrives on molecular imaging techniques that aim at the investigation of the relationship between spatial organization, structure and function of molecules in biological systems. Although impairment in calcium homeostasis, abnormal myocyte energetics and myocardial remodeling have been described to be associated with cardiac dysfunction, the underlying molecular mechanisms involved in the transition from normal cardiac function to heart failure remain poorly understood [27]. Insight into these processes and pathways will be important in the development of new therapeutic strategies for treatment and prevention of heart failure. Under normal physiologic conditions, the heart utilizes fatty acids as its chief energy substrate. Because there is limited capacity for triglyceride storage in the cardiomyocyte, the uptake and oxidation of fatty acids is tightly coupled .The accumulation of triglycerides in the heart, caused by a mismatch between the uptake and the oxidation of fatty acids, is associated with a number of pathophysiological conditions. In animal models (rats) of obesity and diabetes, triglyceride accumulation within cardiomyocytes is associated with impaired contractile function. Although it is unclear how lipids induce cardiac dysfunction, accumulation of intramyocardial triglycerides is associated with altered gene expression [28]. The lipid droplet is endured by a core of lipids, which mostly consists of triacylglycerols (TAG) (90-99%) and to a lesser amount of diacylglycerols (DAG), free fatty acids (FFA), phospholipids and monoacylglycerols. Several distinctive MS peaks and correlated image patterns consistent with the expected oleic/palmitic acid ratio were observed in consecutive SIMS experiments used for the 3D reconstruction of rat heart.

Each molecular pattern in these sections was analyzed in a search for molecular classifiers. For example cholesterol was found to distinguish directly atria from ventricles. The difference in the relative cholesterol content in atria and ventricles was observed in a direct comparison between rat and mouse results (Figure 1 A,C,D). These results corroborate and expand earlier studies performed on heart substructure homogenates that were analyzed with liquid chromatography [29]. Here, for

the first time we visualize the heart cholesterol distribution directly on histological tissue sections without the use of any labeling approach together with a plethora of other molecules. We are not able to assign a molecular structure to the ions at 145 m/z, 175 m/z, 334 m/z, 213 m/z, 969 m/z (Figure 3f,3h,3m,3n,3q) showing a different distribution in the tissue sections. In summary, SIMS is shown to provide extensive local molecular information that complements the conventional histological approaches for the determination of the chemical properties of specific, known anatomical structures inside the heart. As any analytical technique, SIMS has limitations and advantages for molecular histology. In the next section we briefly discuss the most important aspects of SIMS in cardiovascular research.

Limitations of SIMS The extreme surface sensitivity requires careful sample treatment. Sample contamination and molecular diffusion, which can affect the reproducibility of the data, complicate their analysis, or affect the quality of the image, are major considerations in the sample preparation protocols. The multiple molecules present in a tissue section can negatively influence each others' desorption and ionization efficiency and prevent optimal detection. This phenomenon is called ion suppression and can limit the number of detected molecules The SIMS imaging technique is only capable of a semi-quantitative measure of the distribution of elements and molecules in tissue. Absolute quantitation requires the use of isotopically labeled standards. Identification of species is based on single m/z values exclusively as in most SIMS instruments no tandem mass spectrometry can be performed. New instruments will overcome this limitation, but are not yet commercially available.

Advantages of SIMS

SIMS allows for label free molecular imaging of multiple species in parallel directly on tissue. SIMS instruments are used for imaging unknown compounds present in the biological sample without any *a priori* knowledge or labels in a single experiment. It is a true discovery methodology. It has been used for imaging of elemental species in cells at high spatial resolution (~50 nm) and

can typically analyze ions up to 1500 m/z. In the last decade there have been a growing number of studies that show the capacity of SIMS for analyzing biological materials, including different tissues such as mouse brain, human adipose tissue, muscle, kidney and single cultured cells [30-35]. More information on different MS imaging technolgies for biomedical purposes can be found in current review of the various imaging MS technologies for molecular pathology [11]

A major advantage is the reduced amount of sample used; often, after SIMS analysis the tissue surface can be analyzed or stained as if the tissue was pristine. This, in turn, allows the direct comparison with other ex-vivo techniques (e.g. fluorescence and immunostaining) for orthogonal validation of the findings. These techniques are highly specific within the class, but allow the visualization of labeled molecules only with a limited number of detectable analytes per section [36].

Future and perspective:

The application of this method in future studies can be used to identify changing tissue regions that are indicative of human cardiac disease. SIMS improves tissue classification necessary to perform retrospective studies, will assist clinical studies from the bench to bedside, and can guide therapeutic choices. the direct application of SIMS on the same heart tissues as used by pathologists improves and accelerates molecular diagnoses. Molecular tissue classification after SIMS based on known biomarkers or using unsupervised multivariate analyses can positively affect patient treatment. The evolution of a heart disease during treatment can be monitored based on the tissue biomarkers identified by MSI. In cases where traditional biomarkers cannot be clearly detected in biopsies, SIMS could become critical to the outcome. At this point, it is obvious that further clinical studies using SIMS technology are required to fully validate this method. Nonetheless SIMS as well as MALDI-MSI have opened the door to molecular tissue classification, not only for diagnostic and prognostic purposes but also for treatment development. MS based molecular imaging is becoming one of the basic information providers for personalized medicine, especially when used in complement with Magnetic resonance imaging (MRI). A major advantage for SIMS will be its

coupling with positron emission tomography, x-ray, computed tomography instrumentation, and MRI for both preclinical and clinical research. The complementarities between non-invasive techniques and molecular data obtained from SIMS-MSI will result in a more precise diagnosis of the "molecular state" of a living system.

In clinical studies, the need for information on the spatial localization of pathologically gene-encoded products has become more pressing. The three-dimensional volume reconstructions generated by SIMS-MSI data now offer the possibility to compare the molecular data with data obtained using positron emission tomography or MRI [37]. These multi-modal molecular imaging approaches will strengthen the fundaments of molecular imaging research.

Conclusions

The SIMS imaging approach can be used to detect and probe the molecular content of tissues in an anatomical context. Anatomical atlases based on optical images are widely used for anatomical and physiological reference. A series of secondary ion images obtained from successive tissue sections of rat heart can be used to produce a 3-dimensional (3D) molecular reconstruction that contains both pieces of information. SIMS provides detailed high resolution molecular images of tissue surfaces. The results reported here represent the first 3D molecular reconstruction of rat heart by SIMS imaging. The measurements were extended on mouse and human heart samples. Human tissue analysis is demonstrated to benefit from the potential of SIMS imaging for the investigation of the distribution of elements and biomolecules directly on the surface of cardiovascular tissues.

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Figure legends

Figure 1. (A),(B1),(C), Metal-assisted SIMS images of a sagittal section of three different rat hearts; (A1) anatomic differences from rat heart visualized with H&E staining after SIMS analysis. (B,B1) SIMS-MSI images of ions detected from representative rat heart valves and H&E stained tissue sections. (D) Metal-assisted SIMS images of a sagittal section of mouse hearts (datacube images), (D1) H&E stained tissue sections and (D2) spectrum of SIMS.

Figure 2. High-resolution (8192x8192 pixels) SIMS total ion images in black and white and complementary H&E stained images showing the different morphological structures of the heart observed in both molecular imaging modalities.

Figure 3. Metal-assisted SIMS images of a sagittal section of a rat heart. Top image shows the spatial distribution of ions (a,b,c,d) in the aorta wall. For instance, the image e and i shows distribution for the main cholesterol ions (m/z 369 and m/z 385) that are localized to the aorta wall, aorta valve, right coronary artery and right and left atria whereas are not observed in both ventricles. The image f shows the ion at 145 m/z very localized in pulmonary artery, right atria and atrioventricular valve. Interestingly, m/z 175 and m/z 213 are observed only in ventricles. The bottom image m, in contrast, shows m/z 334 localized only in the pericardium The image q shows the high spatial distribution of at 969 m/z in both atrias, aorta wall, left atrioventricular valve and right coronary artery. All ion image scale bars = 100 μm.

Figure 4. (A) PCA spectral results after VARIMAX optimization show a strong contribution for among others cholesterol ([M-H_2O+H]+ at m/z 369.1 and [M-H]+ at m/z 385) and choline (m/z 104) showing correlation with aorta wall, left and right atria, semilunar valve, atrioventricular valve, left ventricle, right ventricle and coronary artery. The distributions based on the PCA results (B) results in a molecular underpinning of the different areas that morphologically identified with H&E staining (C). The intensity of each ion is indicated in the color chart on the left from white (high) to dark (absent).

Figure 5. Workflow of the 3-D recontruction. (A)Tissue section, (B) Gold deposition is done by sputter coating, (C;C1) SIMS- ToF Mass Analyzer, (C;C2) mass spectral peaks, (C;C3) datacube images, (D,E) reconstruction of the heart showing 3D-spatial distributions of three different ions: 145 m/z (red), 23 m/z (green) and 39 m/z (blue), (F) co-registration of forty individual MS imaging.

Figure 6. (A) Metal-assisted SIMS images of human heart. All the images obtained are from the some section. All ion images scale bar = 100 μm. (B) H&E staining. (C) Section stained with sirius red. Connective tissue stains red. In this trichrome stained specimen (image D), collagen is colored blue and smooth muscle is red. The images B, C and D illustrate a trasverse-section of left ventricle muscle, clearly include an intra-myocardial blood vessel to illustrate the staining pattern in gross sheets of cardiomyocytes that can be seen towards the periphery of the figures.

Online supplementary supporting material:

Figure 1. (A) Sectioning a rat heart. Blockface images of the cryomicrotome during the sectioning process. Scalebar is 2.5 cm. Figure (B). Metal-assisted SIMS images of sagittal different sections of the rat heart. All images show the some spatial distribution of sodium (m/z 23).
All ion image scale bars = 100 μm.

Figure 2. The distributions based on the PCA (A,B,C) results in a molecular underpinning of the different areas of three different sections of human sample. PCA spectral results: A1, B1 and C1. The intensity of each ion is indicated in the color chart D from white (high) to dark (absent).