Grant Proposal Guide

for SpatialOMx

Innovation with Integrity

SpatialOMx

Outline

Abstract

MALDI Guided SpatialOMx® represents a quantum advance in how tissue is analyzed. It combines a label-free imaging technique that delivers sensitive distribution mapping for both targeted and untargeted compounds, ranging from metabolites to lipids to proteins with regionally targeted 4D-Omics analysis. The timsTOF fleX is the premier SpatialOMx tool – integrating a high-speed MALDI Imaging source with the fastest and most sensitive platform for 4D-Omics, from the well-established timsTOF Pro, into a single high-performance mass spectrometer. SpatialOMx improves on conventional solution-based Omics approaches by providing the same molecular depth while also retaining spatial information critical to gaining insight into disease development and treatment. CCS-enabled 4D-Omics, based upon TIMS and PASEF® technologies, provides unmatched selectivity, specificity, and throughput for MALDI. The integrated workflow delivered by the timsTOF fleX provides researchers with the best tool for deep dive into regionally targeted biochemical diversity associated with tissue state, in a single platform offering improved operational efficiency.

How to use this guide

This guide is intended to provide basic knowledge and convincing reasoning for the utility of Bruker's MALDI Imaging and SpatialOMx methodologies, including a broad selection of images for use in your grant proposals. MALDI Imaging has well defined capabilities that are outlined herein, as well as technical details that are unique to the comprehensive software and instrumentation that we offer. The data are presented in either a "big picture" workflow manner or a "pick and choose" tile view for you to construct your own tailored graphic. Please utilize the materials here however you can, from taking the text to mixing and matching graphics to tell the story that is important to you.

Tips to maximize success of your proposal

Why MALDI Imaging is an innovative approach

MALDI Imaging is a new form of molecular histology that can complement existing imaging techniques by providing label-free mapping of thousands of compounds across a broad range of molecular classes. Correlating molecular distributions and changes in expression within a tissue provides a deeper understanding of proteomics, metabolomics and lipidomics of disease pathology.

In pharmacology research, that spatial context is utilized in tracking drug molecules and their metabolites within a tissue sample. Visualizing the location of these molecules and tracking their metabolic fate is crucial to understanding a drug's pharmacokinetics and pharmacodynamics and its resultant efficacy and toxicity. Such data allow direct comparison among drug compound candidates, their delivery system right to the disease site and ultimately their therapeutic efficacy - information that is invaluable in reducing ambiguities, time and costs within the drug discovery processes.

For basic biological research and clinical applications, the importance of spatial context has been manifested in various existing imaging techniques including optical or electron microscopy and even magnetic resonance imaging. Unlike immunohistochemistry, a form of molecular histology offering very high molecular specificity, MALDI Imaging is a label-free and multiplexed technique, allowing for the detection of hundreds, if not thousands, of ions simultaneously. This inherent characteristic allows MALDI Imaging to amass distinct mass profiles across a tissue section, that can be data-mined for biomarker discovery while still retaining specific molecular detection. As such, it allows concomitant exploration of correlated or vertically integrated molecular relationship across large cohorts.

In combination with the power of data analytics, Bruker's innovative software solutions advance untargeted analyses without the need for *a priori* knowledge. Advancements in disease diagnostics and translational research are enabled by Bruker's comprehensive solutions that go beyond just instrumentation.

Broader impacts of SpatialOMx

SpatialOMx is the integration of MALDI Imaging to identify and target specific regions in a tissue section for deeper 4D-Omics analysis. MALDI Imaging has the added benefit of identifying spatially significant regions based on molecular phenotype, though it lacks the thorough molecular coverage provided by 4D-Omics. By combining these two into a single strategy, MALDI Guided SpatialOMx characterization of tissue allows researchers to directly characterize molecules that are changing and map where those changes originate within tissue. Studies that will benefit from the enhanced specificity of SpatialOMx are any tissue-based Omics studies where the goal is to discover and characterize molecular changes in tissue microenvironment, including those associated with cellular transformation or disease pathways.

No other single platform offers the analytical performance and flexibility of timsTOF fleX at comparatively lower capital cost and space requirements than similar multi-system configurations.

Benefits to local research environment

SpatialOMx provides new tools for mapping molecular changes within tissues and will have significant impact on Omics studies of cellular processes and disease progression at the tissue level. The ability to map, determine, and identify molecular changes in a regionally specific manner will be unique to the local research community. Having a single platform such as timsTOF fleX that delivers both state-of-the-art MALDI Imaging but also 4D-Omics analysis brings maximum efficiency to the laboratory.

Enable collaboration

Enabling the SpatialOMx workflow in your research environment will provide an opportunity to establish collaborations between disciplines including, but not limited to, pathology, biology, and radiology. The SpatialOMx workflow is unique in bridging the divide between MALDI Imaging and traditional Omics research and provides a valuable pathology context for molecular changes occurring in tissue specimens. Pathological expertise will, therefore, be essential to utilize the full value of this novel method. Acknowledging these collaborative opportunities and providing details on how the working relationship will be enabled can increase the success of your grant application.

Section 1: Getting started with MALDI Imaging

Integrating MALDI Imaging into your workflow is straightforward and can yield high value spatial information to many different fields and research applications involving tissue samples.

For the drug discovery or metabolic scientist

As most drug molecules and small molecule metabolites are not inherently fluorescent, research focused on metabolic processes or pharmacokinetic-pharmacodynamic relationships are at a disadvantage with respect to fluorescence microscopy techniques. A targeted or untargeted approach to drug or metabolite localization in tissue can uncover new relationships between biochemical pathways and disease state and add information to liquid biopsy measurements. Moreover, the operational cost of each measurement is often < 100 USD which allows for MALDI Imaging to be deployed often and at earlier points in the discovery pipeline. Castellino *et al*. have summarized the innovation that MALDI Imaging brings to the pharmaceutical industry, which also extends to any small molecule study. In addition, quantitation using MALDI Imaging opens new opportunities for more complete studies, as demonstrated by Swales *et al*.

The metabolome is the final manifestation of biochemical pathways and encompasses an extremely large variety of structural classes produced from the breadth of cellular processes. Changes in the metabolite composition reflect the outcome (phenotype) of interactions at the genomic, transcriptomic, and proteomic levels. The additional spatial context afforded from MALDI Imaging is a cornerstone to a deeper insight into variations in cell metabolism as a result of diseases, therapeutic interventions, environmental influences, etc.

For the clinician/pathologist

Much of the research effort in the clinical sphere is driven primarily by the goal of improving patient outcome. However, a clinician's work is a constant struggle against ambiguities where decisions are often made based on incomplete information. Whether in diagnosis or in treatment, any improvement in discerning a patient's clinico-pathological picture can lead to a better outcome.

Bruker's MALDI Imaging instruments are uniquely suited for interrogating biochemical information in substructures of tissue sections, making new areas of study accessible, such as complex cancer biology and its myriad biochemical interactions. MALDI Imaging has been shown to provide deeper information on patient-derived samples by providing important spatial context molecular changes. MALDI Imaging is poised to be an indispensable component in the clinician's toolbox, with the potential for improving patient stratification, personalized treatment regiments, and even early diagnosis.

To illustrate these points, a recent study by Berghmans *et al.*, using MALDI Imaging revealed that neutrophil defensin 1, 2 and 3 can predict the efficacy of current immunotherapy of non-small cell lung cancer beside PD-L1 expression. Patients with high defensin expression at the tumor margins showed better response to immunotherapy, allowing clinicians to identify patients that will most benefit from such treatments. Such studies have direct impact on patient outcome and are highly translatable into clinical sphere.

For the Omics researcher

Liquid chromatography (LC), in conjunction with tandem mass spectrometry (MS/MS), is the core technique of Omics strategies and yields a higher dynamic range for detection than MALDI Imaging with small sample amounts. Rendering tissue specimens for LC-MS/MS analysis involves homogenization and extraction in which spatial context about individual cells are lost. More and more, LC-MS/MS scientists are realizing the intrinsic value of spatial context in identifying cellular contributions to molecular changes and MALDI Imaging is an important tool to recover molecular information in the spatial dimension.

Microextraction techniques, such as laser capture microdissection (LCM), retain spatial information for subsequent proteomic and metabolomic measurements. MALDI Imaging, with its label-free assessment of tissue, provides a level of cellular specificity for identifying specific cell phenotypes for microextraction.

For the Lab Director

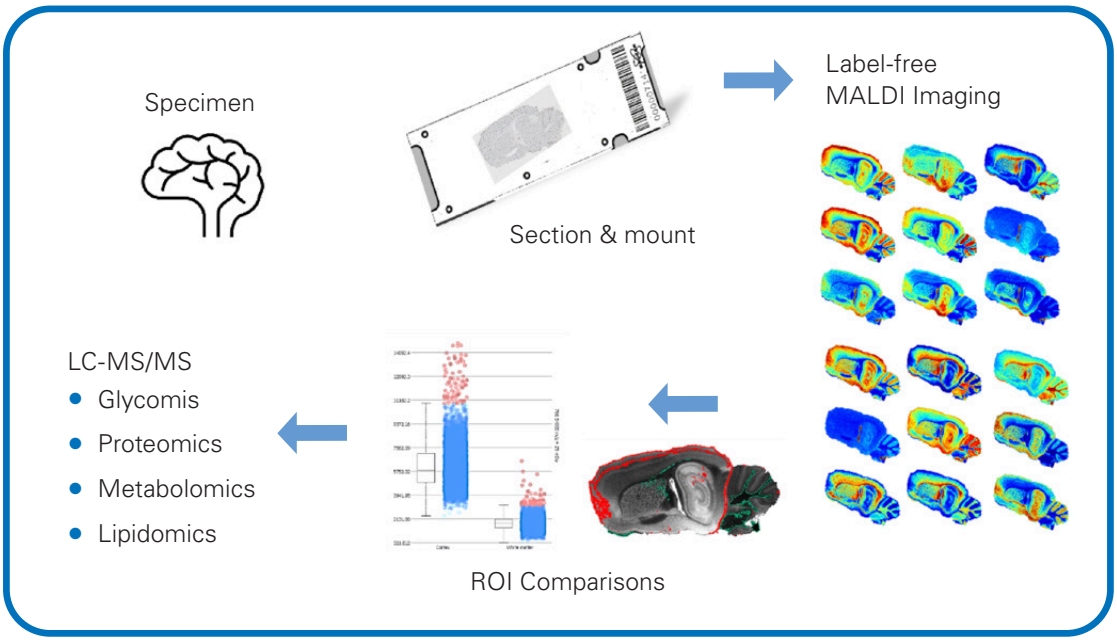
The timsTOF fleX is designed to provide maximum economics while ensuring quality data streams that translate into results. With both MALDI Imaging and 4D-Omics LC-MS/MS capabilities in a single instrument, timsTOF fleX has lower capital cost and space requirements than similar multi-system configurations. Switching between ionization modes is instantaneous for maximum system utilization.

A full line of consumables and software tools are available to ensure data reproducibility and accuracy. IntelliSlides® provide an optically transparent substrate that is amenable to MALDI Imaging and microscopy along with permanent markings that are used by SCiLS™ AutoPilot software for automated measurement setup and instrument quality checks.

Section 2: SpatialOMx and label-free molecular imaging

MALDI Guided SpatialOMx Workflow

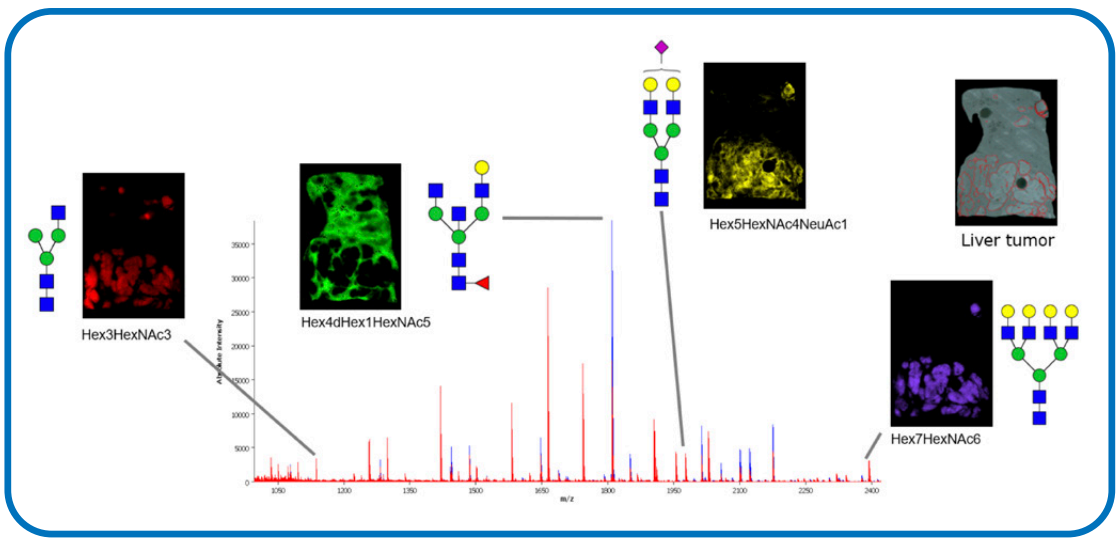
Using MALDI Imaging to highlight molecular subtypes to be analyzed using 4D-Omics, is a novel workflow that enables the deepest molecular insight from tissue with spatial context. MALDI Guided SpatialOMx brings together high resolution MALDI Imaging with 4D-Omics capabilities of timsTOF fleX to create a powerful, novel 4D-Omics workflow targeted to specific regions in the specimen. First, MALDI Imaging is used to identify and categorize sub-populations, or molecular phenotypes, spatially across a tissue. These regions are subsequently targeted for analysis at high dynamic range with LC-MS/MS for compound identification – both known and unknown. This new approach provides researchers with unmatched sensitivity for relevant molecular distributions in tissue and is enabled by the high sensitivity of 4D-Omics. SpatialOMx can therefore increase sensitivity and specificity of any Omics research, including proteomics, lipidomics, glycomics, and metabolomics.



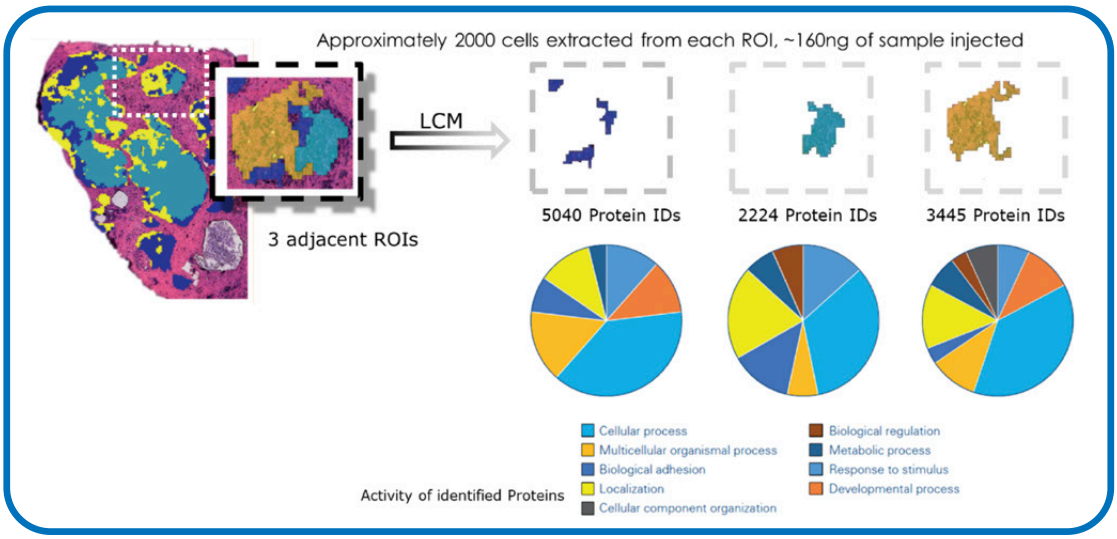
Overview of MALDI Guided SpatialOMx workflow

SpatialOMx represents a shift in how tissue is analyzed when studying molecular changes associated with disease development and treatment. Over the past decade, numerous Omics approaches have offered new insight into molecular mechanisms occurring within and across the complex network of cells that make up tissue. Unfortunately, because of their solution-based approach, these techniques are largely unable to correlate specific molecular involvement to distinct cell phenotypes or localized regions within the tissue. Additionally, such approaches tend to dilute signals of interest by analyzing extracts from the tissue rather than extracts from the few cells of interest. What is needed is a new methodology for analyzing tissue that provides the same molecular depth as traditional Omics workflows but which also retains the spatial context of those signals within the cellular network. SpatialOMx provides researchers with the ability to map molecular distributions in tissue *in situ*, identify regions of interest that express the desired molecular profile, and selectively target these subpopulations for 4D-Omics analysis.

Conventional molecular imaging methods such as immunohistochemistry (IHC) or fluorescence imaging rely on tagging the target molecule to facilitate detection. Antibody or molecular tag selection requires that the target compound be known in advance, thereby limiting the use of these techniques for non-targeted discovery experiments. Antibodies can be specific but have limited applicability beyond visualizing protein distributions, leaving the method blind to many key compound classes such as metabolites and lipids that are active participants in cell signaling and metabolism. SpatialOMx is an unlabeled imaging technique that provides sensitive distribution mapping of both targeted and untargeted molecules, ranging from metabolites to proteins.



N-Glycan imaging of prostate tumor illustrating regional specificity of individual glycans. Image courtesy of Dr. Richard Drake, Medical University South Carolina.



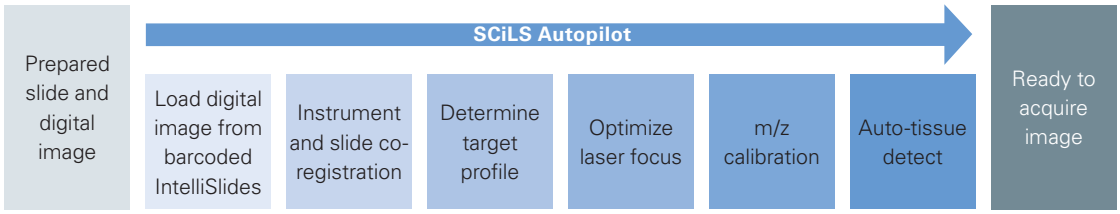
Lipid imaging of breast cancer reveals three adjacent lipid subtypes which are extracted by laser microdissection for 4D-proteomic analysis. Thus, lipid subtyping enables deeper proteomic phenotyping.

Ease of Use

One of the limitations of MALDI Imaging is the relative complexity for setting up experiments due to the disparate technical nature of sample preparation and measurement for microscopy and mass spectrometry. However, an advanced degree in MALDI Imaging is no longer necessary to acquire high quality data. A full range of consumables including fleXmatrices and IntelliSlides take the guesswork out of preparing samples for imaging. FleXmatrices are pre-proportioned for automated sprayers and tagged for analyte class to be measured.

IntelliSlides are permanently etched with barcode and registration fiducials which also provide visual mounting areas for tissue sections. A companion spray mask ensures the sprayer applies matrix only to tissue sections to be measured.

The new SCiLS Autopilot software is designed to work with IntelliSlides to simplify and expedite the experimental set-up, instantly elevating beginners to expert users.



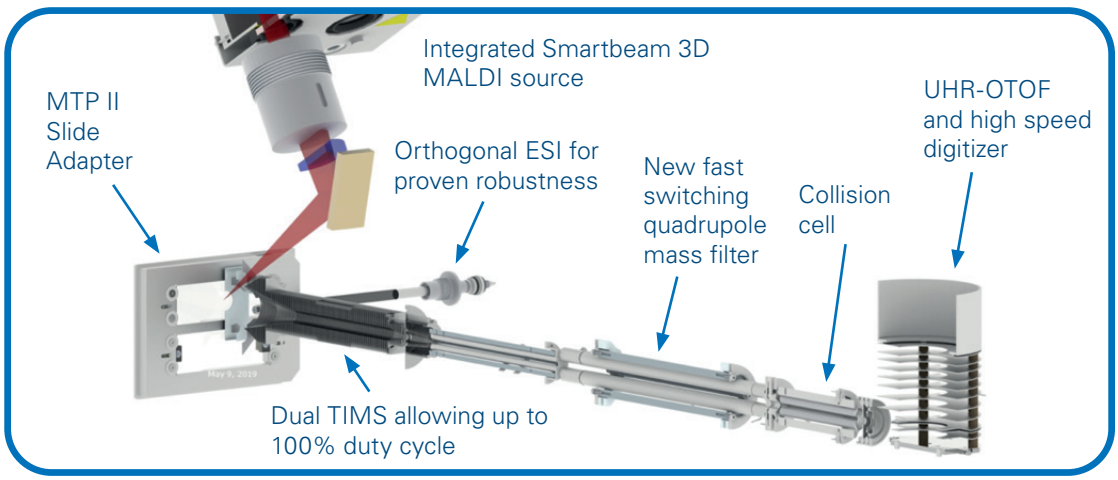
Section 3: Platform capabilities and performance

The timsTOF FleX is the only system capable of both targeted and untargeted MALDI Imaging and 4D-Omics, with key instrument and software components supporting these workflows. Specific features are highlighted in this section with relevant graphics.

SpatialOMx Instrumentation: timsTOF fleX

Key system features:

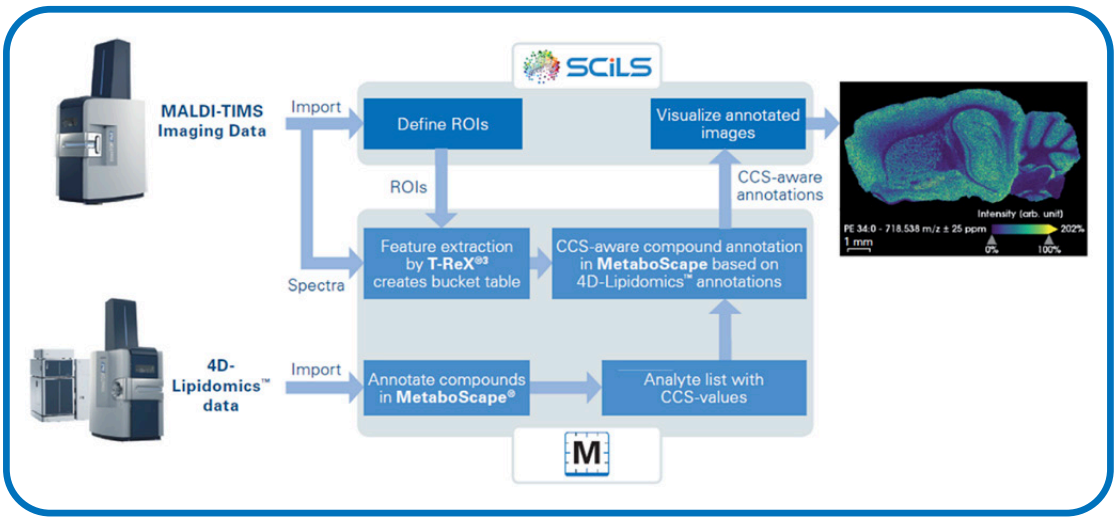
- **Dual MALDI Imaging and LC-MS/MS Platform**: Combines the established 4D-Omics functionality of the timsTOF Pro, with high-performance MALDI Imaging capabilities in a single instrument.
- One-click 'instant' switching between LC-MS and MALDI operation imposes no downtime.
- **TOF Mass Range**: 20-40,000 *m/z*
- **Mass Resolution**: 60,000 at *m/z* 1221
- **Trapped Ion Mobility (TIMS) technology**: Provides accurate and reproducible CCS values from first principle. Allows for 'preconcentration' of ions, significantly boosting sensitivity and allowing very low sample loading.
- **Parallel Accumulation – Serial Fragmentation (PASEF)**: Synchronizes TIMS separation with MS/MS precursor selection, allowing >100 MS/MS spectra/second.
- **Collisional Cross Section (CCS) measurements**: Provides unmatched selectivity and sensitivity. TIMS separation of isobaric compounds:
  i) improves detection sensitivity by eliminating chemical background.
  ii) enables clean MS/MS fragmentation of precursor ions isolated by CCS and *m/z*.
  iii) provides crucial 2nd intrinsic molecular identifier for added confidence when identifying compounds.
- **True-pixel imaging**: Analyzing tissue in discrete areas so that measured molecular features can be unambiguously linked to specific cells.
- **Smartbeam 3D Laser**: 10 kHz capable of "zoom mode" 5-15 μm true pixel imaging
- **Robust**: Lower sample loadings mean less cleaning and higher reproducibility.
- **MALDI-2**: Optional configuration that maximizes sensitivity



CCS-enabled SpatialOMx

Just as retention time (RT) is an analyte attribute within the liquid phase, the gas-phase structure of an analyte is also reproducible and intrinsic. The timsTOF fleX measures Collisional Cross Section values using Trapped Ion Mobility Spectrometry (TIMS), which can achieve IMS resolution up to 200, equivalent to a drift tube length of over 2 meters. Using first principles, CCS can be quickly determined for small molecules, metabolites, lipids, peptides, and protein molecules. Since they derive from first principles, CCS values from TIMS is fully compatible with published CCS determined from drift tube measurements. Since CCS is intrinsic to the molecule, accurate measure/prediction in either MALDI or LC-MS mode increases accuracy and confidence in assigning molecular identification.

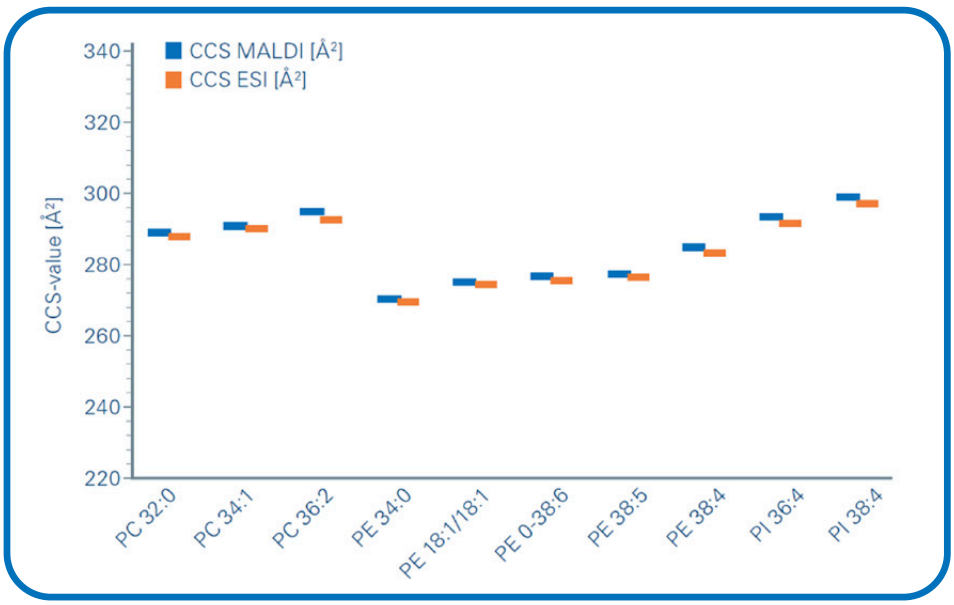
This new 4th dimension can also be used to selectively analyze ions of unique interest within the CCS space. Ions having similar or even identical *m/z* but separated by CCS can be selected independently for MS/MS analysis, adding additional confidence in annotation assignments and quantification completeness.



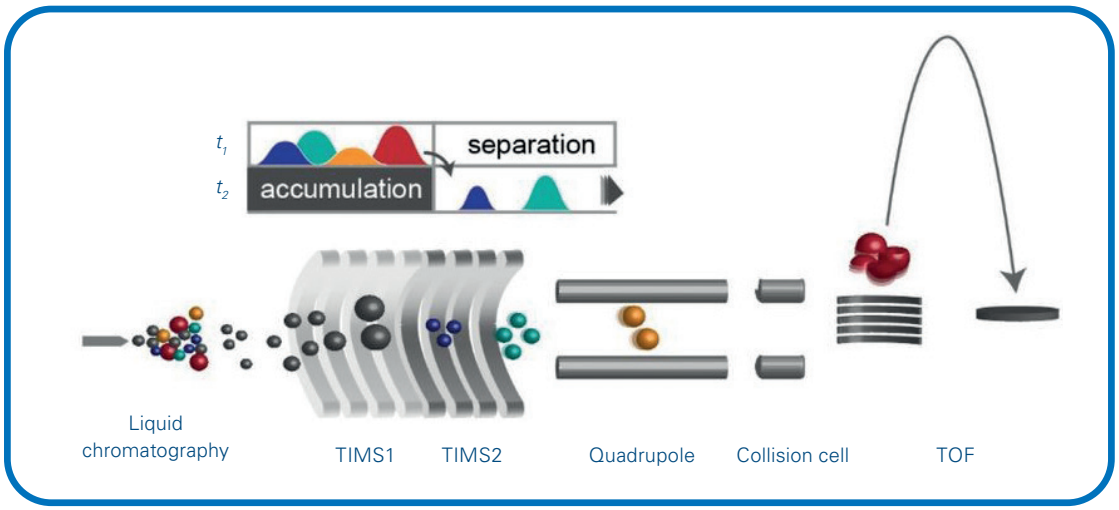
Schematic overview of the CCS-enabled SpatialOMx workflow for lipids. Features are extracted from 4D-Lipidomics data, annotated in MetaboScape® and exported as an Analyte List with CCS-values. MALDI Imaging data is imported to SCiLS™ Lab, where regions of interest (ROIs) are created. Spectral and region information are then used in MetaboScape® for automatic annotation of the MALDI Imaging compounds based on the ESI lipidomics Analyte List. CCS-values serve as additional validation criterion for the annotation. Finally, annotated MALDI images are visualized in SCiLS™ Lab.

TIMS Sensitivity Boost

SpatialOMx measurements, MALDI or 4D-Omics, typically involve very small amounts of sample that demand the most sensitive mass spectrometer. Samples from laser capture microdissection, small organs, and even single cells all require very high sensitivity. The dual-TIMS funnel in the timsTOF fleX series significantly improves sensitivity while maintaining unprecedented sequencing speeds. Whether eluting from a column or resulting from multiple laser pulses, ions enter TIMS1 as a temporally diffuse ion cloud of mixed *m/z* and CCS. Over a duration of 20-100 ms CCS separation occurs by the opposing forces of constant gas flow and an electrical gradient. The collection of CCS-separated ion packets are then transferred to TIMS2 while TIMS1 begins filling with a new ion cloud. Ions are eluted from TIMS2 towards the downstream TOF analyzer by sequentially lowering the electric field to elute ions by CCS. Thus, ions enter TIMS1 spread over a 20-100 ms accumulation window, but elute from TIMS2 as discrete ion packets separated by CCS and compacted into 2-5 ms segments before entering the TOF. This temporal pre-concentrating effect of dual-TIMS funnel results in up to a 30x signal-to-noise improvement compared with other continuous acquisition instruments. Simultaneously filling TIMS1 while eluting from TIMS2 delivers 100% duty cycle.



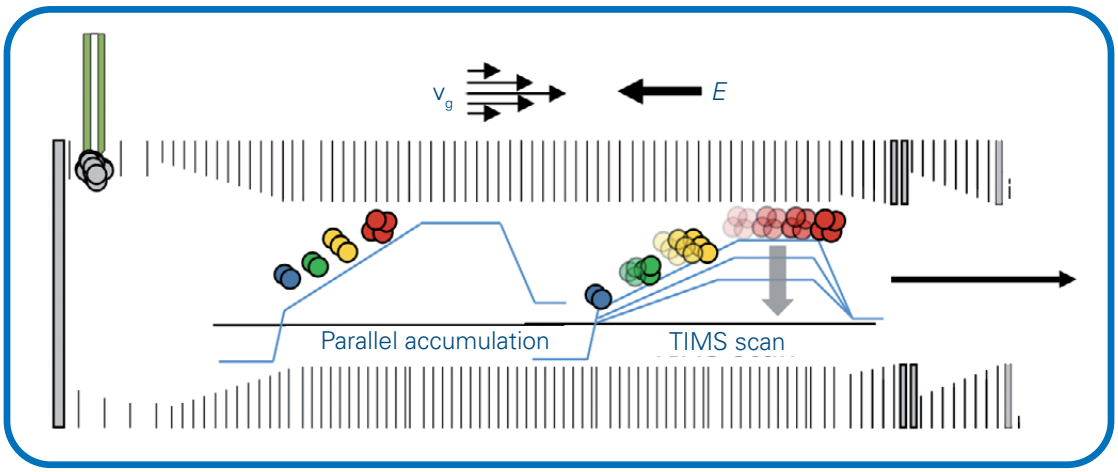
Reproducibility of CCS-values across ESI- and MALDI-ionization for different lipids.



Ions separated by CCS elute from TIMS cell at different times rendering fragmentation spectra specific to ions of a specific CCS and m/z.

Parallel Accumulation - Serial Fragmentation (PASEF)

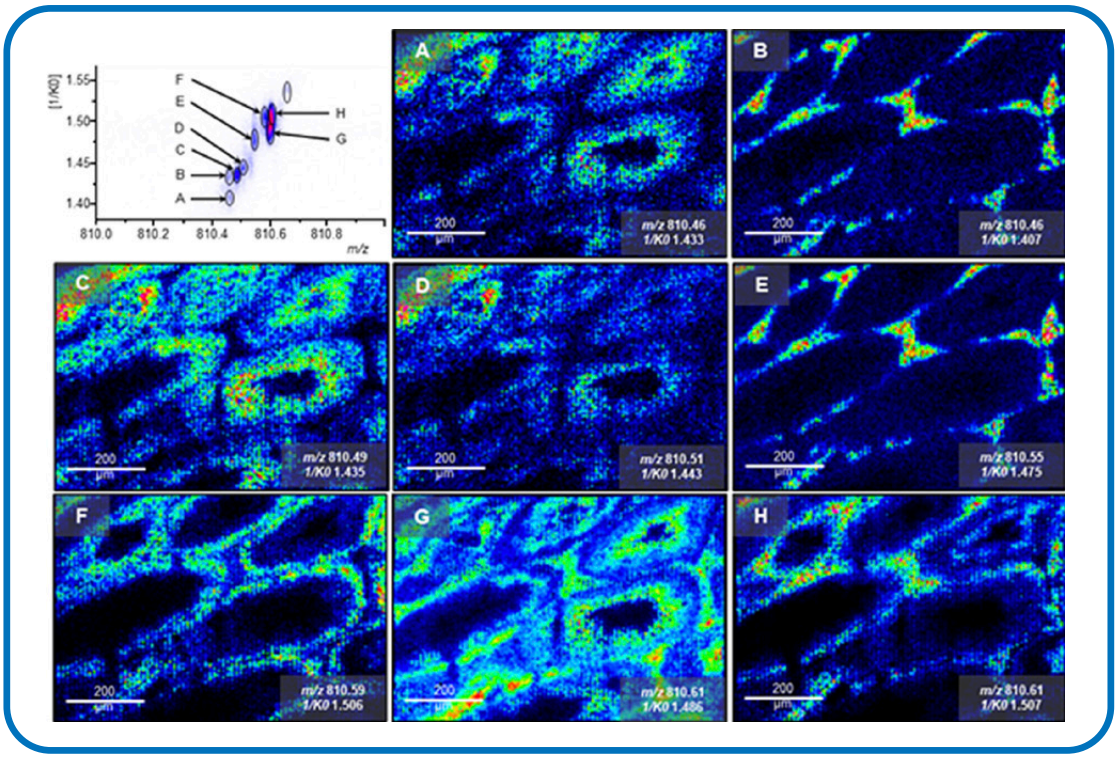
The patented PASEF acquisition mode is only available on the timsTOF Pro and timsTOF fleX series of instruments. PASEF synchronizes MS/MS precursor selection by the quadrupole with the elution of ions from the TIMS cell to maximize the number of fragmented precursors per TIMS scan, thereby increasing the sequencing speed several-fold. The standard PASEF method acquires on average 120 MS/MS scans with a duty cycle of 1.1 s, achieving an MS/MS acquisition rate of >100 Hz. The sensitivity boost provided by the TIMS time-focusing combined with 100% duty cycle, allows PASEF to overcome the traditional problem of sampling fewer ions as acquisition rate increases.



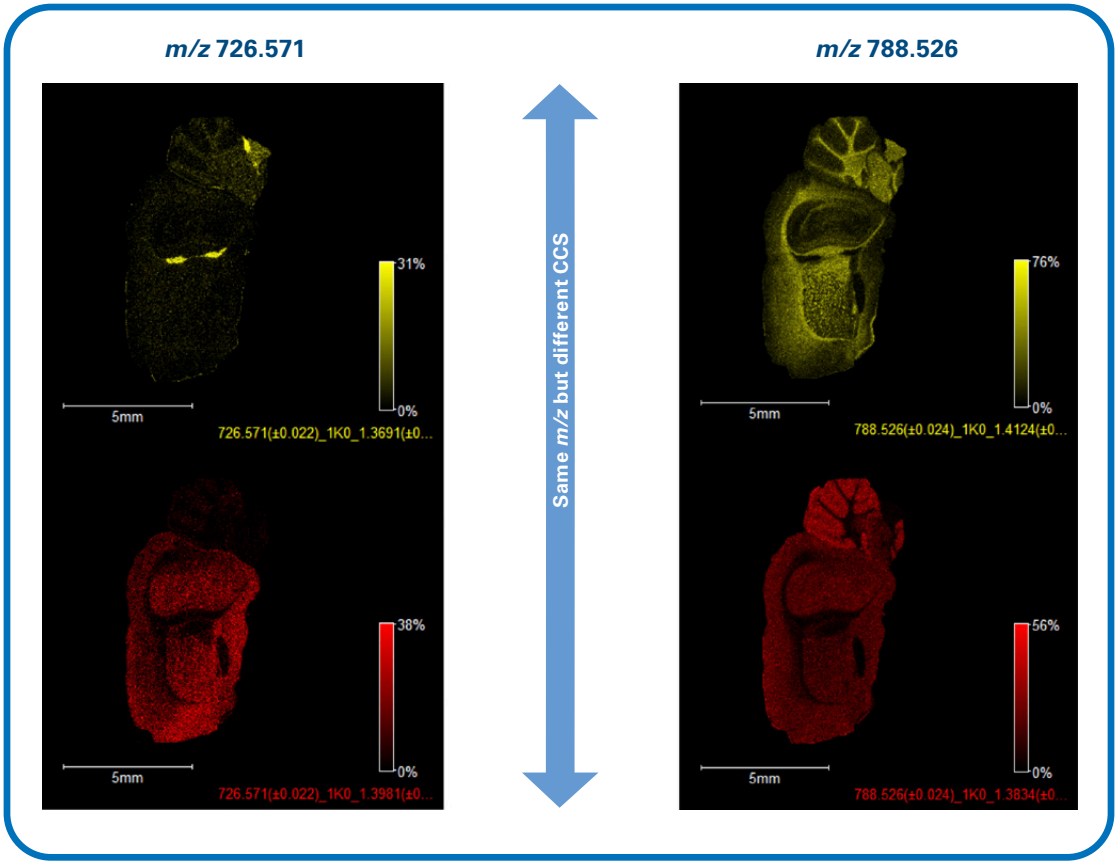
The dual-TIMS cell accumulates and separates in TIMS1 while the previous packet of ions elutes from TIMS2 to provide 100% duty cycle.

TIMS Imaging

Given the molecular complexity of biological specimens TIMS separation delivers obvious advantages for MALDI Imaging applications. Unlike solution-based analyses, chromatographic separations are not amenable to MALDI analysis direct from tissue and it is often not possible to separate the multitudes of isobaric and isomeric MALDI ions solely relying on mass resolving power. TIMS provides an orthogonal tool for separating similar isobaric and isomeric ions by combination of CCS and *m/z*. In fact, with maximum TIMS resolving power of more than 200, TIMS provides the only tool with the capacity to resolve and image many isomeric species.



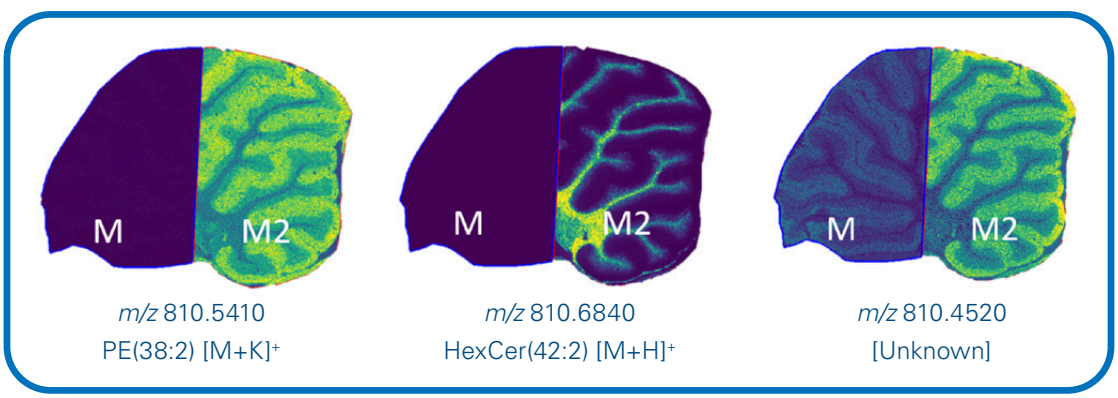
CCS-resolved isobaric images from rat testis section.



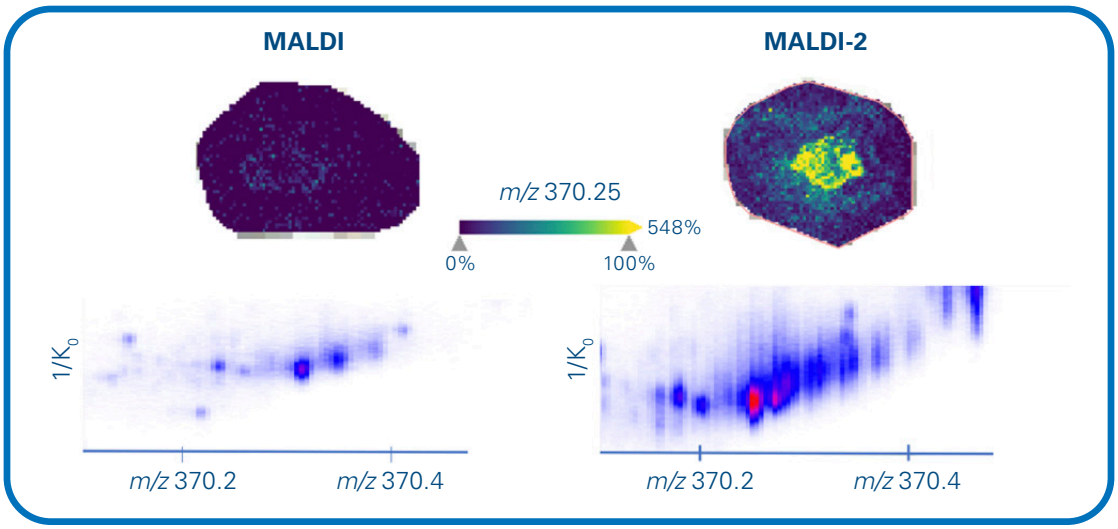
CCS separation reveals isomeric molecular images.

Use MALDI-2 for maximum sensitivity

Innovations in ionization, such as MALDI-2, push the range of detectable compounds even further. In MALDI-2, a second laser is utilized for post-ionization of molecules inside the MALDI plume a few microseconds after its formation. Resulting post-ionization enhances sensitivity of many compounds by as much as 1000 fold but also enables the detection of compound classes not detectable by traditional MALDI.



Enhancement of lipid signals in the rat cerebellum by MALDI-2 (right half) compared to traditional MALDI Imaging (left half).

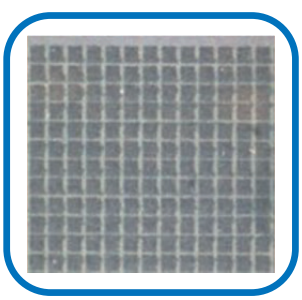


MALDI-2 enhancement of cholesterol (minus neutral water) in the canine heartworm. Heat maps illustrate the enhanced signal strength and complexity of the MALDI-2 data that is further benefited by TIMS separation.

High-speed, high-spatial resolution MALDI Imaging with True-Pixel resolution

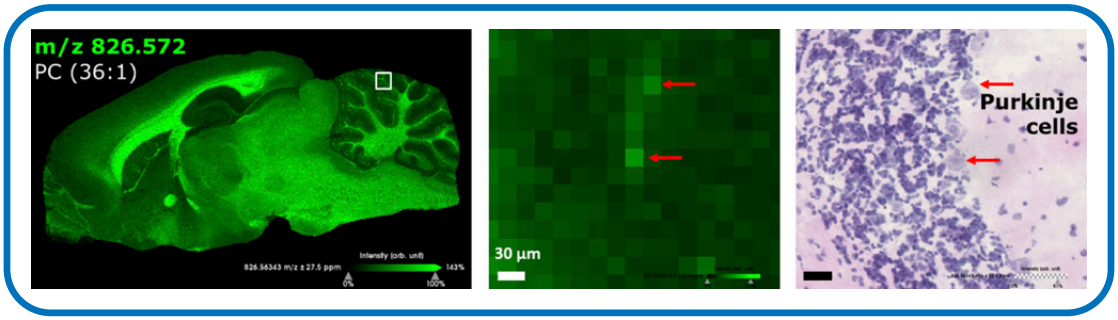
A primary goal of an imaging study is to detect changes in the biochemistry of cell transformation or to correlate changes in molecular expression to disease cells. In order to accomplish this, the image should be acquired by measuring molecular features from discrete cells. Only MALDI Imaging can capture molecular fingerprints from specific regions of a sample. Continuous scanning sources acquire data from both sides of a pixel boundary, 'smearing' the signal from a discrete cell cluster into multiple pixels in the image. It is therefore essential that detected signals be associated with a single unique area of the sample, so called true-pixels, where ion intensity originates from specific cells.

A large body of work in scientific literature validates using MALDI Imaging to investigate many molecular classes such as glycans, lipids, metabolites, proteins and products of on-tissue enzymatic digestion or chemical derivatization. The timsTOF fleX is capable of imaging these compound classes at high spatial and mass resolving power, while also offering True-Pixel imaging whereby the cellular origin of detected signals can be unambiguously determined to a discrete AND unique cellular region. Constant ionization sources such as DESI cannot provide true-pixel imaging. With these sources, a portion of an observed signal likely originated from one or more areas of sample adjacent to the pixel area of the image.



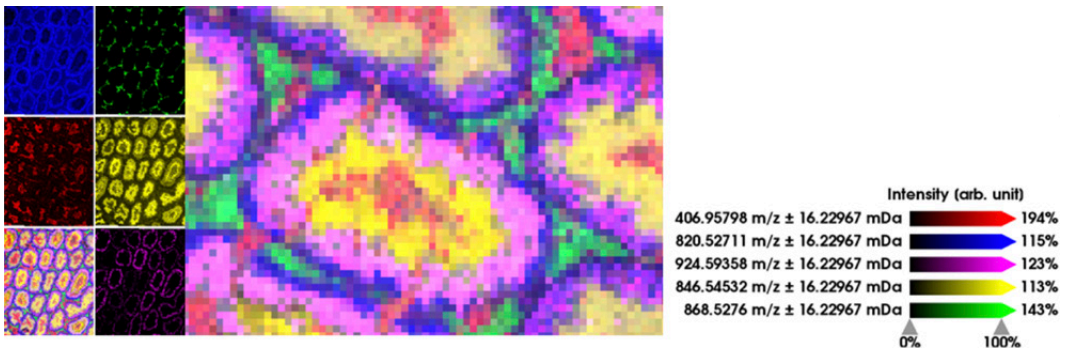
True-pixel imaging - detected signals are unique to the area of sample defined within boundaries of the visualized pixel. Only with true-pixel imaging can one confidently determine that 100% of the detected signal originated from a discrete region of sample. True-pixel imaging is not possible with constant scanning techniques such as DESI

Only true-pixel imaging can reveal small architectural features in tissue.



Ions localized to individual Purkinje cells in rat brain

Spatial resolution: Specified 20 µm resolution; laser diameter 5 µm

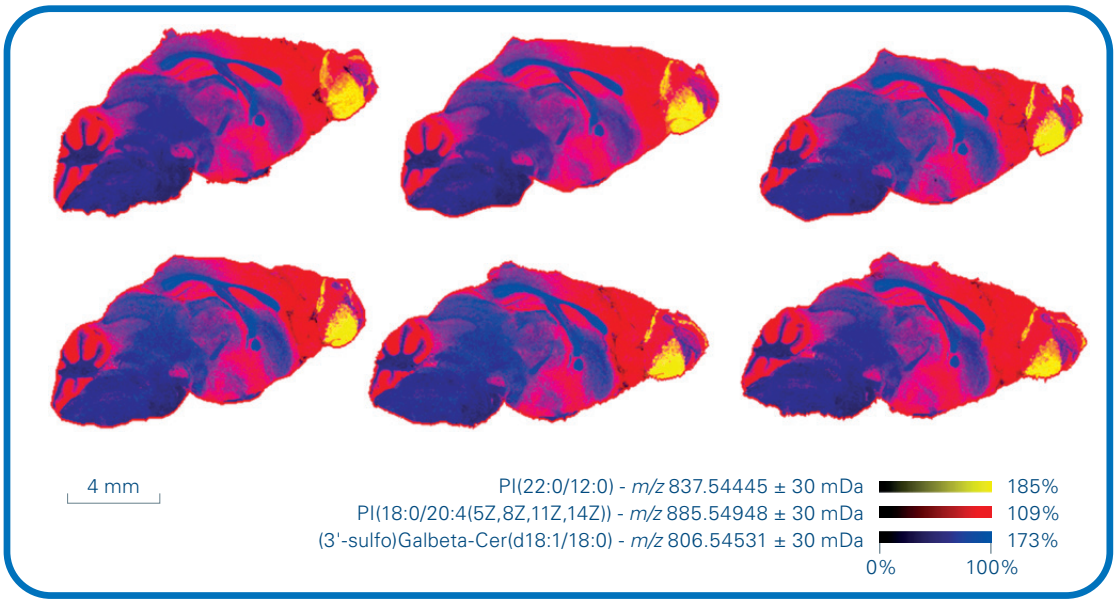


Ions localized to wall and lumen of tubules in rat testis.

timsTOF fleX reproducibility and robustness

The timsTOF fleX exhibits excellent robustness and reproducibility for both LC-MS Omics and MALDI Imaging of large sample cohorts, a quality essential for long-term clinical research. The extremely sensitive orthogonal LC-MS ion optics of the timsTOF fleX requires less sample throughput. The contamination of the instrument is minimized, resulting higher stability and more reproducible results.

Below are three superimposed phospholipid images from six serial mouse brain sections at 20 μm pixel resolution. Each image is composed of approximately 125,000 pixels (~2 hours measurement time) for a total of 12 hours of continuous MALDI measurement. The distinct similarity of the lipid images demonstrates the robustness of timsTOF fleX to matrix contamination and reflects the reproducibility of MALDI performance.



Lipid images from 6 rat brain sections acquired in sequence shows high stability of signals over time.

Section 4: SpatialOMx software solutions

A single SpatialOMx dataset can contain hundreds of thousands of mass spectra. Manual evaluation of data is not feasible, and successful interpretation of such large data sets requires computational data mining strategies.

SCiLS Lab

Bruker provides sophisticated statistical imaging software, SCiLS™ Lab, specifically for analyzing molecular information in spatial context. SCiLS Lab offers numerous statistical tools such as comparative analysis of multiple samples (classification, co-localization analysis, PCA, ROC, and spatial segmentation for automatic data analysis) and interactive 2D and 3D visualization. SCiLS Lab also integrates with Bruker's MetaboScape software for annotating MALDI Images with high-confidence compound IDs from MetaboScape. SCiLS Lab is available as Multiple Vendor Support (MVS) version, enabling users to combine MALDI Images from different platforms using the universal imzML data format.

Histology – QuPath

Histology is a standard tool in pathology that relies on visual examination of morphological differences between cells of the tissue. Differences in cell count, morphology, and other factors guide the diagnoses of tissue-based diseases. In a sense, histology offers an after-the-fact perspective of cellular transformation and offers very limited information about the molecular mechanisms that fuel cell transformation. As such, histological examinations can be highly subjective and often indeterminant, particularly when used to identify cells at a particular stage of transformation. Conversely, molecular expression can provide a more specific differentiator for cells presenting similar morphology and enables predictive insight into what the cell 'intends' to do, and on what time frame. SpatialOMx utilizes MALDI Imaging to map molecular expression across the tissue, and subsequently, segment sub-structure expressing similar molecular phenotype.

Segmentation mapping

The basis of SpatialOMx is to analyze a series of molecular images and categorize regional similarities, or molecular phenotypes, often where histology is unable to differentiate. SCiLS Lab examines the molecular fingerprint within each pixel of data and clusters them into 'segments' based on their molecular similarity. A color-coded dendrogram is then created, with each branch representing populations of each segment. A color-coded spatial map is also generated, with each segment overlaid onto the digital image of the tissue. The automatic segmentation pipeline is fast and requires only a few mouse clicks and offers a clear molecular histology view of the section. Segmentation is ideal for untargeted analyses to quickly determine what molecular features are commonly represented within a given cellular architecture.

Once generated, individual segments or molecular subtypes may be selected for further statistical analysis and comparison to render molecular features at a high degree of cellular specificity. Further, for SpatialOMx analysis, coordinates of segments can be transferred to the laser microdissection device for harvesting cells from specific molecular subtypes.

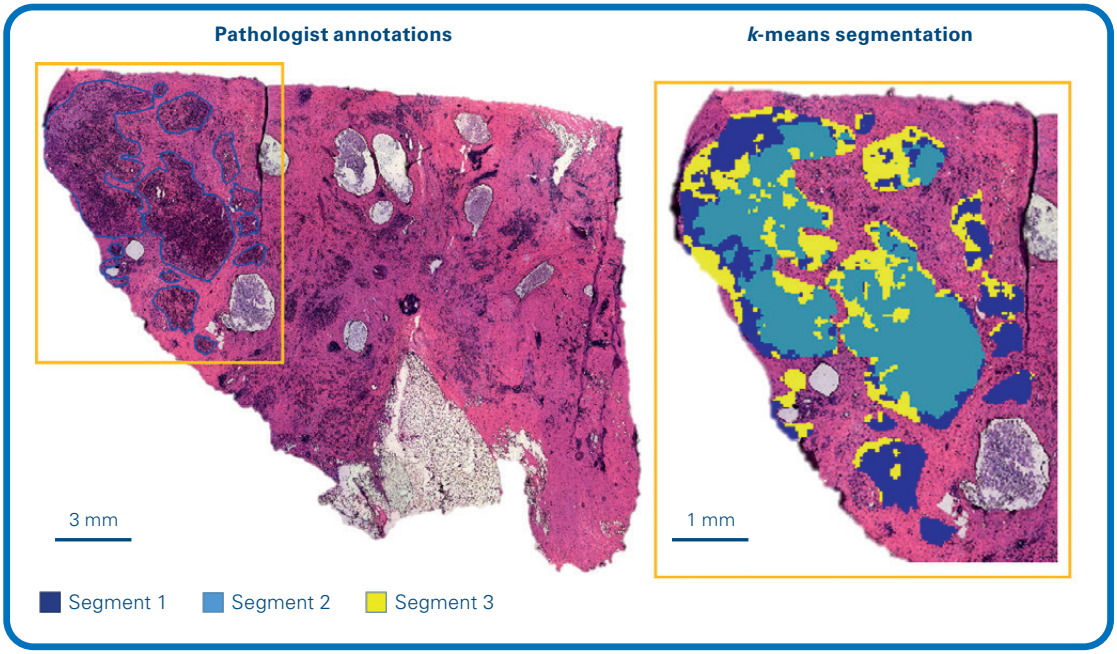


Image of the H&E stained breast cancer section with tumor area marked in blue by a pathologist (left side). Zoom on the tumor area after segmentation analysis using k-means clustering, which reveals three molecular tumor subpopulations (right side).

MetaboScape

MetaboScape® is the ideal tool for 4D-Lipidomics™ and 4D-Metabolomics™ using timsTOF fleX. Even for smaller molecules, PASEF delivers high sample throughput and sensitivity. Further, the ability to separate isobaric compounds that have similar retention time in the 4th dimension, CCS, and to extract and identify key molecular feature differences between sample groups is invaluable for clinical research. Even at shorter LC run times, TIMS separates co-eluting isobaric and isomeric compounds. For MALDI Imaging, MetaboScape offers CCS-enabled database searching of *m/z* and CCS for more confident molecular annotations without MS/MS. When reference CCS values are missing from the database, CCSPredict algorithm is available for accurately confirming lipid structures with high confidence.

Section 5: Additional example data

Sample data that is included in this section:

A Lipid imaging in rat brain

B Lipid imaging in rat testis

C N-glycan imaging in heartworm

D N-glycan imaging in human liver (sample courtesy of Peggi Angel and Richard Drake, MUSC) and subsequent LCM

E Proteomics LCM (app note)

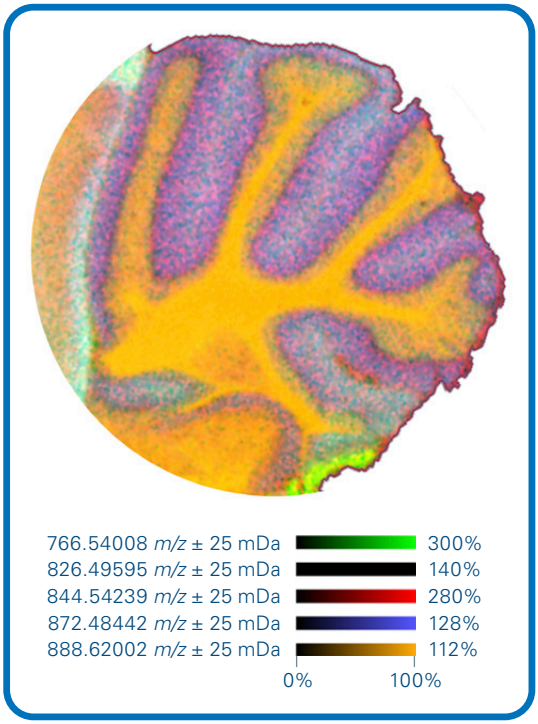
F Small molecule imaging in mouse eye

G Small molecule imaging in zebrafish (OSU)

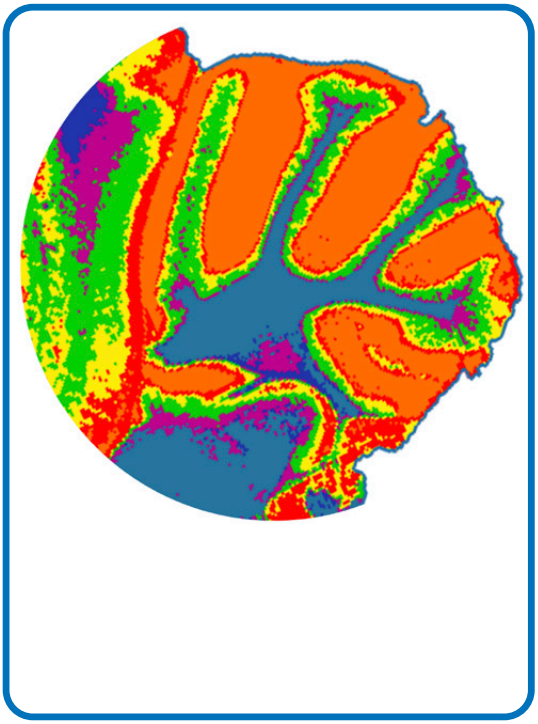
H Quantitation enhancement provided by TIMS

Example A: Rat brain lipid imaging

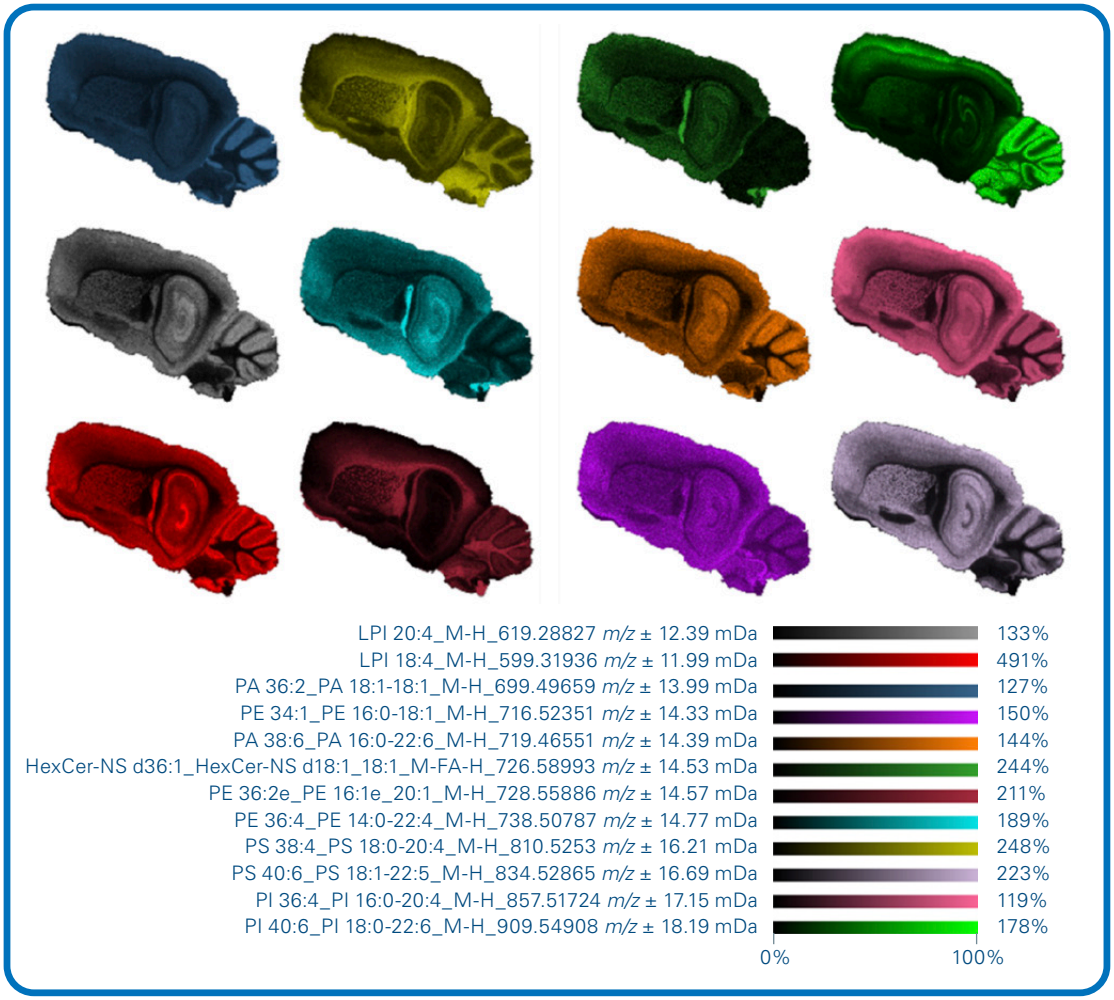
Sample description: A sagittal rat brain tissue section (10 µm thickness) was imaged at 20 µm lateral spatial resolution in negative ion mode (from *m/z* 300 to 1200) on the timsTOF fleX with TIMS on.



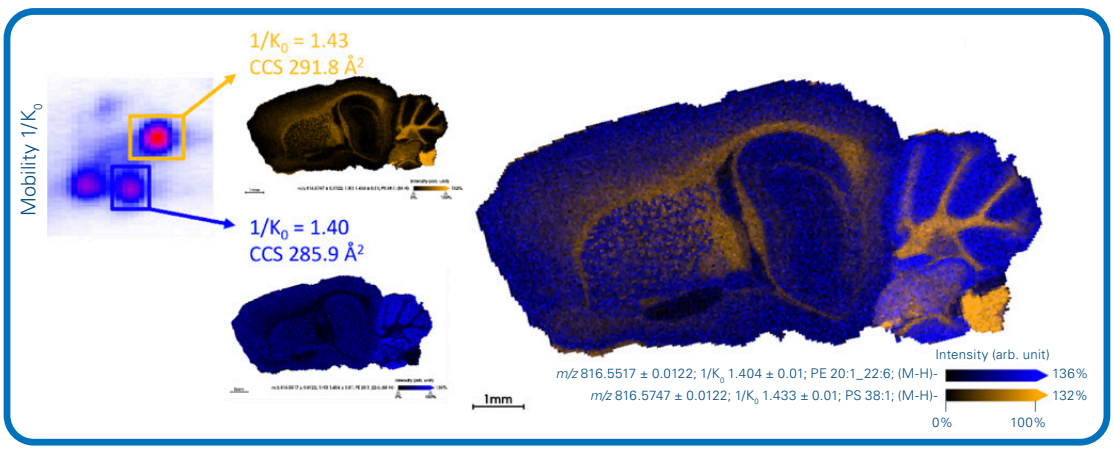
Expression of multiple lipid images in the rat brain, with clear definition of cerebellar tissue.



Segmentation mapping, a form of hierarchical clustering, is utilized to view molecular signatures that follow cell phenotype. The molecular fingerprint can be isolated using statistical analysis with no a priori knowledge of the biological context.

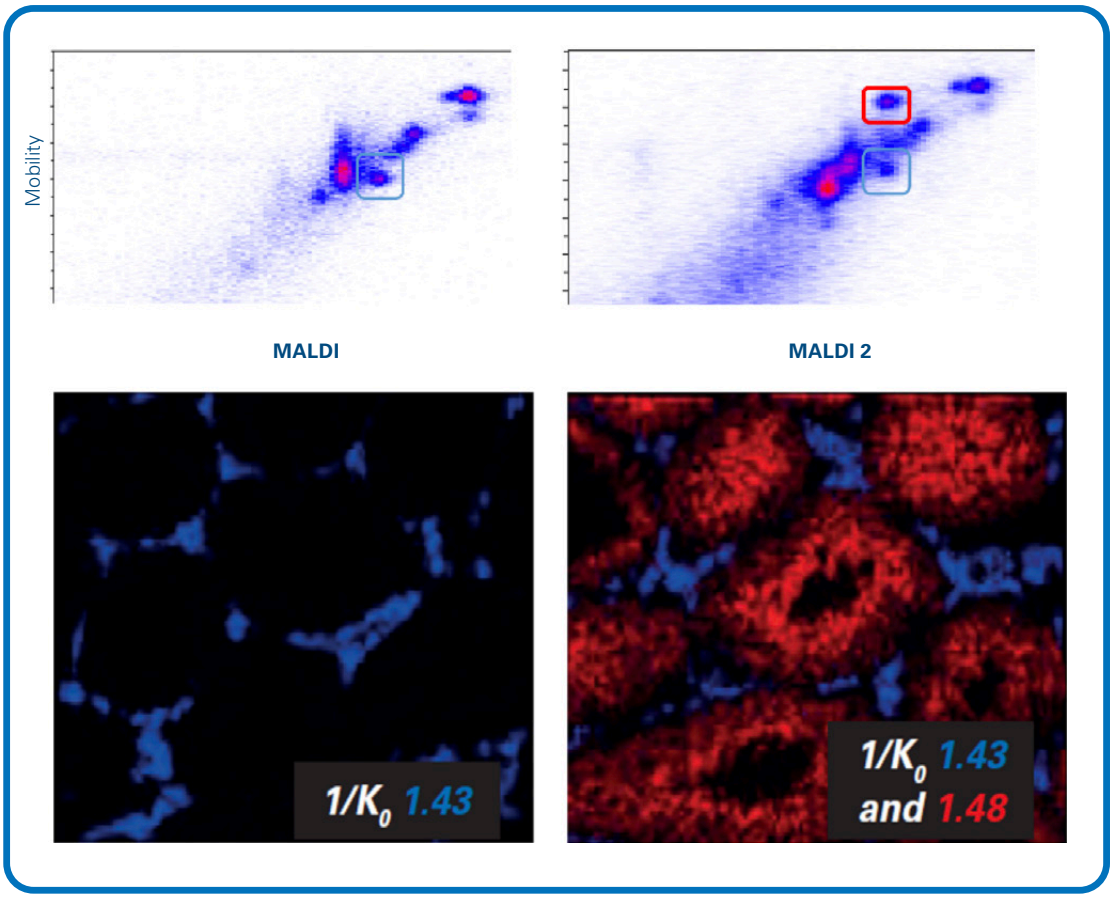


A selection of ion images from rat brain are shown with the corresponding annotations that results from the MetaboScape software.



TIMS separation of species that would be unlikely to be resolved by mass alone are shown for rat brain. Generating CCS-enabled images are part of the data analysis workflow in SCiLS Lab.

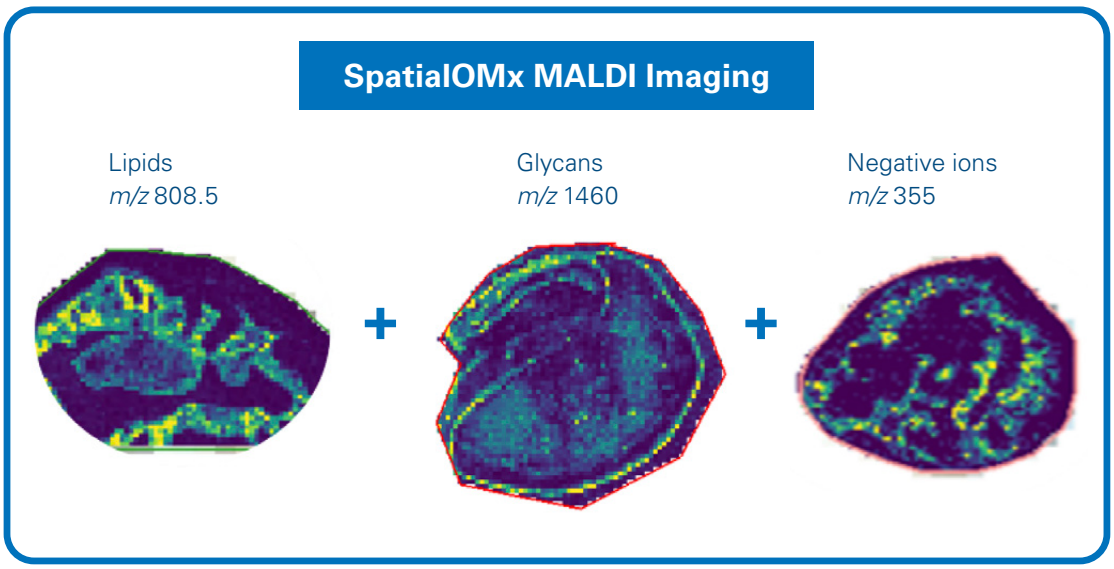
Example B: Rat testes lipid imaging



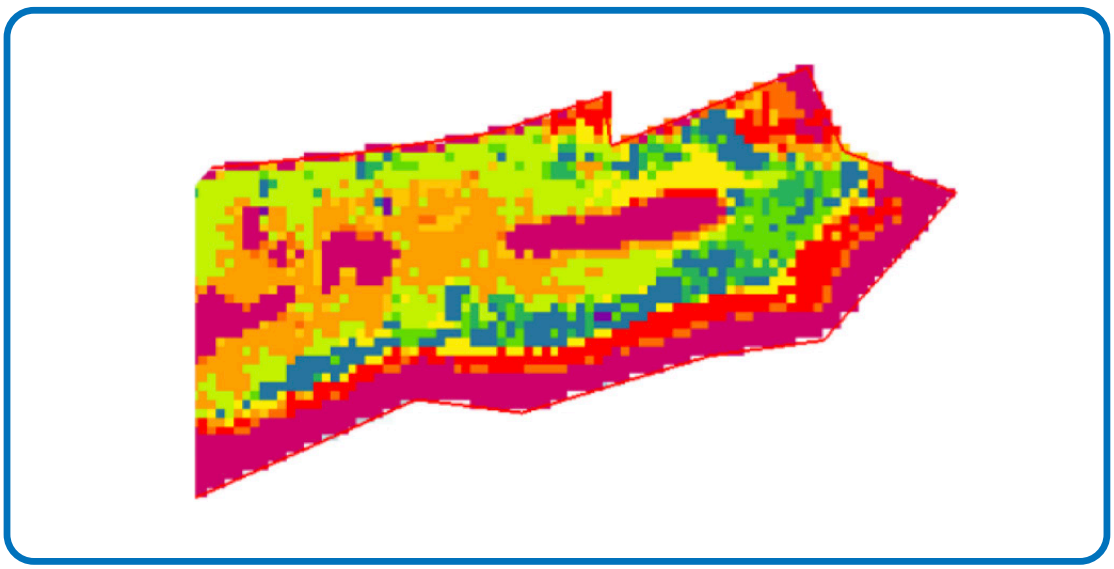
MALDI-2 ionizes additional species from tissue and TIMS allows visualization of each mobility separated peak of interest.

Example C: Heartworm glycan imaging

Sample description: *Dirofilaria immitis* tissue sections (10 µm thickness) embedded in carboxymethylcellulose (CMC) were imaged at 10 µm lateral spatial resolution in positive ion mode (from *m/z* 150 to 1600) on the timsTOF fleX with TIMS on, after processing with PNGase F to release glycans. Data courtesy of New England Biolabs.



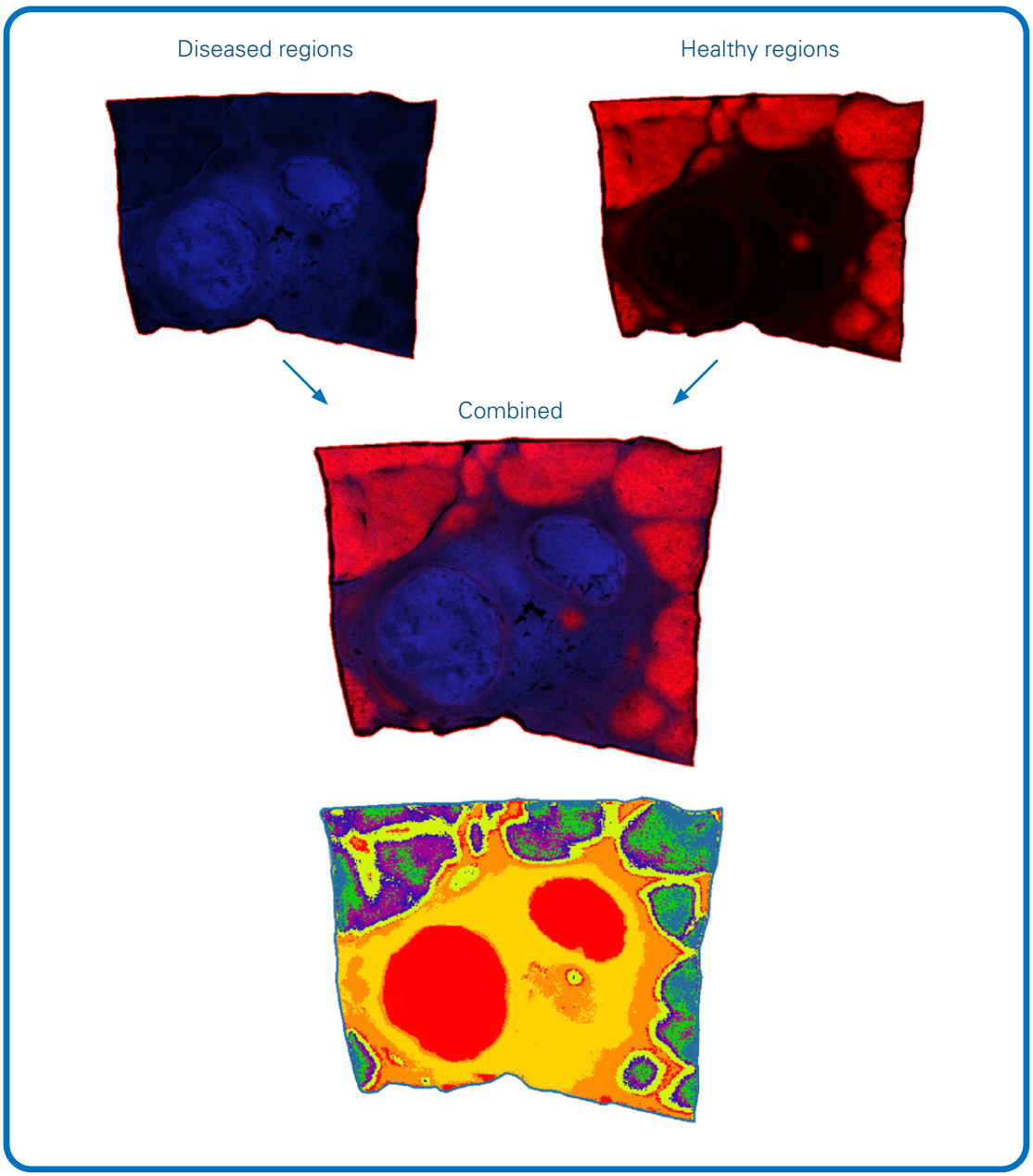
MALDI imaging of tissue cross-sections of canine heartworms provides multiple dimensions of information, including lipids, glycans, and small molecules.



Segmentation analysis of canine heartworm (processed for lipid analysis) that reveals distinct cellular layers within the cuticle that express a different lipid profile.

Example D: Human liver glycan imaging

Sample description: Human liver carcinoma FFPE embedded tissue section (10 µm thickness) was processed with PNGase F and was then imaged at 80 µm lateral spatial resolution in positive ion mode (from *m/z* 0 to 4000) on the timsTOF fleX. Data courtesy of Peggi Angel and Richard Drake at the Medical University of South Carolina.



Example E: Protein – LMD workflow

Sample description: Human fresh-frozen breast tumor was sectioned (12 µm) and mounted on polyethylene napthalate (PEN) for MALDI Imaging at 50 µm lateral spatial resolution from *m/z* 300-1600 followed by LMD.

Sample data:

Regions of the desired molecular phenotype are identified and located in the segmentation image. In the example illustrated below, ~2000 cells were removed from each of three selected regions using laser capture microdissection (LCM). The excised tissue was then extracted and digested for bottom-up 4D-Proteomics™ analysis on timsTOF fleX. Rather than deconvolve signals from complex homogenate into regional origin, the resulting identified proteins unambiguously originated from the excised regions, providing a window of very high cellular specificity for the pathways each protein participates, only possible using SpatialOMx.

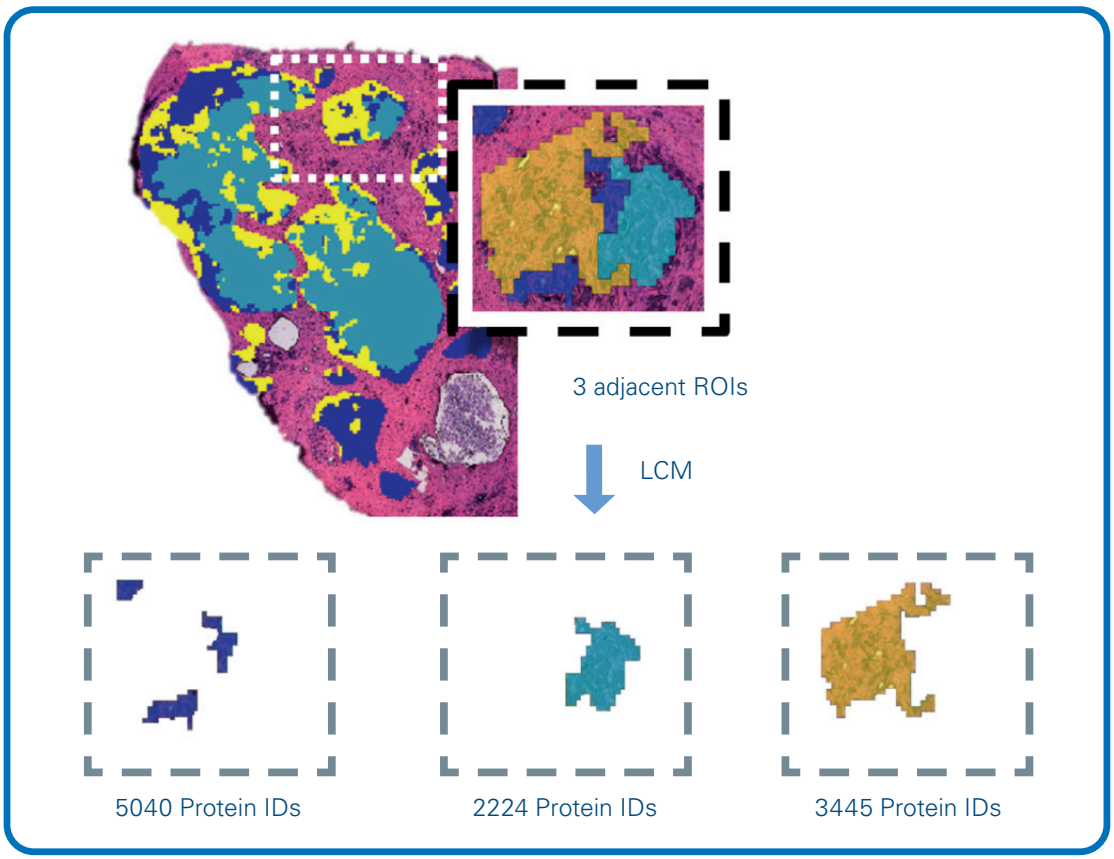
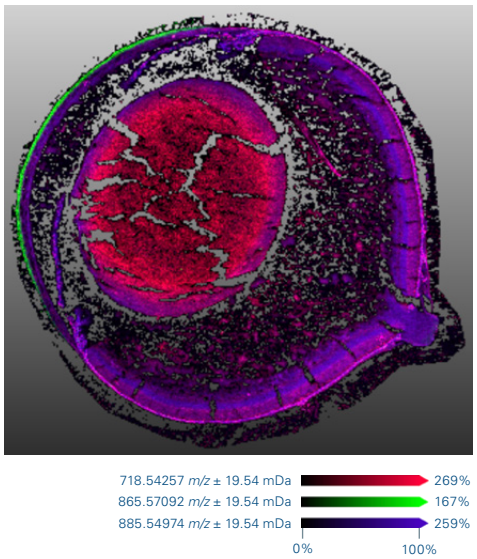


Image processing pipeline to produce ROI boundary information as x,y-coordinates for laser capture microdissection from segmentation raw data. The workflow includes smoothing, removal of small objects, holes filling, boundary detection and up-scaling to the optical image.

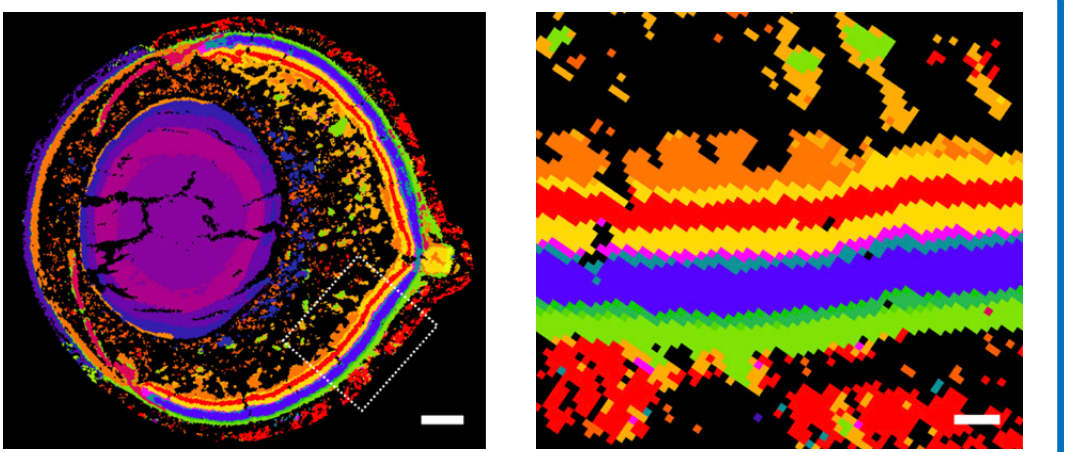
Proteomics of the three excised tumor subpopulations (segmented from imaging data) reveals different biological process characterization based on the identified proteins. Only SpatialOMx delivers such a high degree of regional specificity for biological activity. Proteins from microdissected tissue (approx. 160 ng) were extracted, digested with trypsin and peptide extracts were run on the timsTOF fleX using PASEF. Number of protein IDs per tumor subpopulation segment and biological process characterization per segment reveals deeper molecular information SpatialOMx provides.

Example F: CNS small molecule imaging

Sample description: mouse eye section imaged in negative ion mode at 10 µm spatial resolution



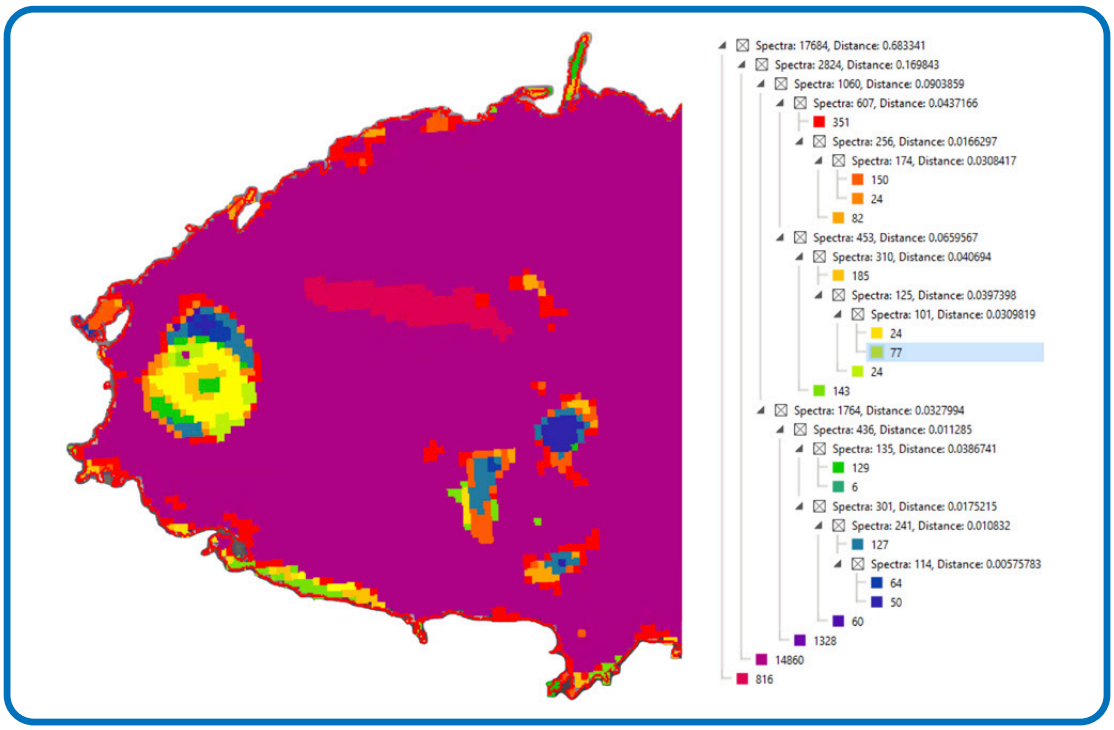
Selected lipid images

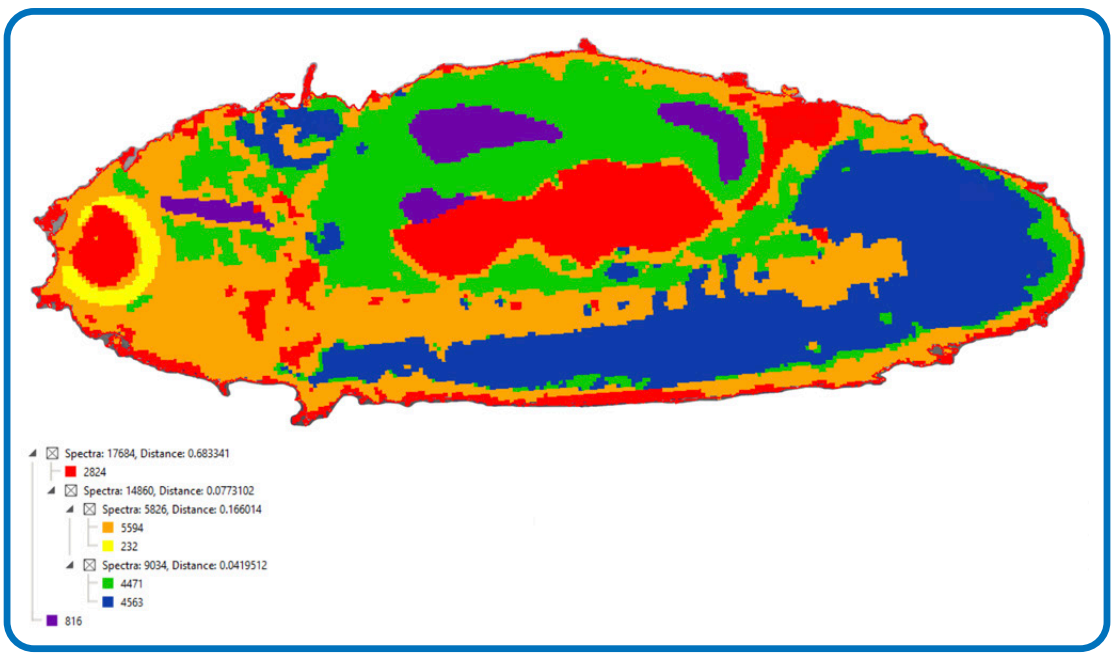


Segmentation image illustrating different cell layers in retina that differ by lipid phenotype.

Example G: Zebrafish

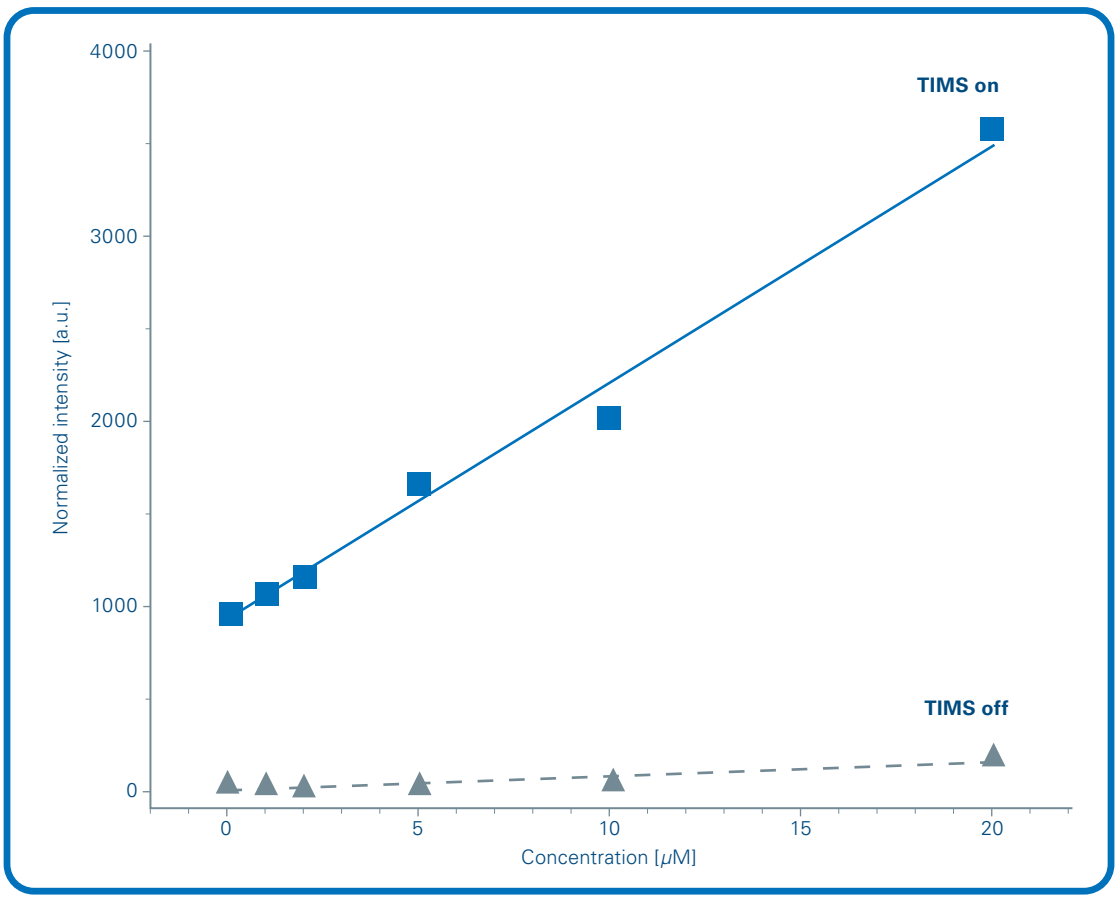
Sample description: Whole zebrafish segmentation images based on metabolite and lipid cluster analysis. Data courtesy of Oregon State University.





Example H: Quantitation

Sample description: Mouse brain homogenate was prepared from control mouse brains for use in the tissue mimetic model. Serial dilutions of MMAE drug were spiked into the tissue homogenate and imaged. MALDI spectra were collected using the timsTOF fleX in positive ion mode with and without TIMS. With TIMS, unresolved isobaric chemical noise from the tissue interferes with computing the peak intensity for the reference. Therefore, TIMS yields a more accurate correlation of intensity with reference spike amount.



Summary: The timsTOF fleX is *the* tool for SpatialOMx

SpatialOMx is the next-generational method for in-situ characterization of tissue, and the ability to map molecular distributions label-free, determine regional bioactivity, and annotate morphological properties will be unique to the local research community. Such an innovative approach will directly impact the research of anyone who is studying cellular processes and disease progression at the tissue level and enable them to lead their fields. Label-free MALDI Imaging is capable of mapping the distribution of more biologically relevant molecules and molecular classes in a single tissue section than immuno-histochemistry or other tag-based imaging techniques. From this data, it is possible to discover cell phenotypes invisible to histology and target selectively for deeper 4D-Omics analysis. The timsTOF fleX is the only tool for SpatialOMx – delivering the fastest and most sensitive platform for 4D-Omics workflows, with a seamlessly integrated high-speed and high spatial resolution MALDI Imaging source. The next generational step for anyone currently doing Omics research, is SpatialOMx.

Relevant Publications:

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Direct N-Glycosylation Profiling of Urine and Prostatic Fluid Glycoproteins and Extracellular Vesicles.
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Appendix

SpatialOMx Protocol:

1. A thin section of tissue is mounted onto an IntelliSlide, and a digital image of the slide is acquired using the TissueScout scanner.
2. A thin layer of fleXmatrix® is applied to the slide using the TM sprayer, which is also available from Bruker.
3. The IntelliSlide is loaded into the timsTOF fleX for MALDI Imaging.
4. User defines portions of the sample to image.
5. MALDI Images are analyzed using SCiLS Lab software, which will aid in identifying spatially relevant molecular changes.
6. Microextractions are collected from the spatially relevant regions of the tissue.
7. Extracted solutions are analyzed on timsTOF fleX for 4D-Omics.
8. 4D-Omics data is analyzed and parsed through appropriate databases for compound identifications.
9. Spatially relevant signals identified by SCiLS Lab are matched to the identified compound list using accurate m/z and collisional cross-section (CCS).
10. SCiLS Lab presents user with a list of annotated images.

Required instrumentation to fully equip a SpatialOMx lab

Execution of a SpatialOMx workflow requires the timsTOF fleX and accessories for both LC-MS/MS and MALDI Imaging workflows. MALDI Imaging sample preparation requires the following accessories: TM Sprayer from HTX Imaging to apply matrix; SpatialOMx Starter Kit, which includes SCiLS Lab Core analysis software, TissueScout slide scanner, IntelliSlides and fleXmatrix. A cryostat and/or microtome is needed for sectioning specimens. For LC-MS/MS analyses, the timsTOF fleX is optimized for use with Bruker's Elute family of LC systems. Most common LC systems are also compatible. Available 4D-Omics software such as PEAKS, MaxQuant, or MetaboScape can be used and depends on the specific area of study.

Instrument training and support

Bruker's extensive network of worldwide Service and Applications teams are available to provide support. Upon installation of the system, an engineer will qualify the system by demonstrating that the system meets all specifications and provides a familiarization training so that users can begin measuring straightaway. Included with each system are certificates for users to attend one of the many training sessions held throughout the year at Bruker's demo facilities. These sessions cover in greater depth components of the SpatialOMx workflows. At any time, Bruker scientists or engineers are available for phone or online support at no charge.

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