Customized rule-based Annotations for Spatial Lipidomics

Introducing automated, rule-based lipid annotations of sulfatide lipid species, leveraging structural properties derived by QCL-MIR guided MALDI Imaging with integrated iprm-PASEF®.

Abstract

Sulfatides are a critical class of glycosphingolipids essential for maintaining axonal integrity, making them highly relevant in neurodegenerative disorder research, including Alzheimer's disease and multiple sclerosis [1]. In Alzheimer's disease, reduced sulfatide levels not only colocalize with amyloid-β peptides [2] but also exhibit reciprocal regulation with amyloid precursor protein processing [3], underscoring their pathogenic relevance. Because of this, comprehensive chemical characterization of this lipid class remains essential for spatial lipidomic analysis. However, the immense structural diversity of lipids makes confident identification a significantly challenging task.

To overcome this bottleneck, we demonstrate a targeted analytical workflow leveraging quantum cascade laser (QCL) mid-infrared imaging guided MALDI Imaging (QCL-MIR guided MALDI Imaging) with integrated imaging parallel reaction monitoring-parallel accumulation serial fragmentation (iprm-PASEF) [4]. This multimodal approach enables the precise determination of structural properties, including collisional cross sections (CCS), for complex lipid classes. By combining structure-CCS-relationship analysis to define subclass-specific three-dimensional (3D) hyperplanes with the deciphering of characteristic and recurring fragmentation patterns for sulfatide subspecies, this methodology provides a highly specific, experimental foundation for lipid identification.

Ultimately, this comprehensive structural data powers automated, rule-based annotations in spatial lipidomics using MetaboScape®. Demonstrated primarily on the 3-O-sulfogalactosylceramide (SM4) subclass, this seamlessly integrated solution enables researchers to transition from manual estimations to precise, reproducible spatial multiomics, significantly increasing both analytical throughput and annotation confidence across complex biological tissues.

Keywords:
timsTOF fleX, iprm-PASEF, MetaboScape, Lipid Annotations, Sulfatides, DataAnalysis, Hyperplane, CCS

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Introduction

Lipids are essential building blocks of cells, forming the structural framework of cellular membranes, including the plasma membrane, organelle membranes, and lipid droplets. Beyond providing structural integrity, they participate in cell signaling, energy storage, and regulation of membrane fluidity and protein function. Chemically, lipids exhibit well-defined properties, including variable chain length, degree of saturation, and headgroup diversity, which determine their overall biophysical behavior. Despite these defined molecular characteristics, the existence of over 45,000 structural conformations, grouped into eight main categories and hundreds of subclasses, makes lipid identification a significantly challenging task in spatial lipidomics.

Although recent advances in on-tissue MALDI Imaging and laser capture microdissection (LMD)-driven liquid chromatography-mass spectrometry (LC-MS) have significantly expanded the coverage of the spatial lipidome, the structural elucidation of lipids remains a cornerstone of modern bioanalytics. Complementary approaches such as trapped ion mobility spectrometry (TIMS) for determining CCS values, together with on-tissue iprm-PASEF on the timsTOF fleX, provide crucial orthogonal information on lipid structure and fragmentation patterns. The integration of ion mobility with mass spectrometry facilitates the confident distinction of isomeric species and enables more accurate annotation of complex lipid compositions within biological samples. Nevertheless, the extensive diversity of lipid species present in a single specimen necessitates automated and rapid workflows for comprehensive lipid coverage, as well as automated annotation software capable of delivering reliable and accurate results across hundreds of molecular features at high computational throughput. Besides metric-based approaches, rule-based lipid annotations offer a unique strategy for consistent and precise identification of lipid species [5,6].

Methods

Materials and sample preparation

Arylsulfatase A-deficient mouse kidney cryosections were prepared at 10 µm thickness on indium tin oxide slides (Diamond Coatings Inc., Gilbert, United States) or IntelliSlides® (Bruker Daltonics GmbH & Co. KG, Bremen, Germany) and spray-coated with 2,5-dihydroxyacetophenone (DHAP) matrix, including an internal standard (SM4 35:1;O2, Avanti Polar Lipids, Birmingham, USA) using a published protocol [3] on an HTX M5 sprayer (HTX Technologies, Chapel Hill, NC).

Mid-infrared imaging guided iprm-PASEF

For elucidating the structural molecular properties of sulfatide species, QCL-MIR guided MALDI Imaging with integrated iprm-PASEF was performed as described previously [1,7]. In brief, mid-infrared imaging data were acquired on a LUMOS II ILIM (Bruker Optics, Ettlingen, Germany) for whole slide imaging in transflection mode using a 4x objective. Subsequent data analysis revealed coordinates for functional tissue areas with known strong sulfatide accumulation, i.e., the inner stripe of the outer medulla (ISOM) and inner medulla/papilla (IMP), for region-focused Imaging (Figure 1A). The guided analytical approach not only enhances sample throughput but also increases the overall depth of molecular characterization. This is evidenced by the construction of a comprehensive sulfatide database derived from iprm-PASEF measurements, which serves as the foundational dataset enabling customized, rule-based annotations within MetaboScape (Figure 1B).

After data visualization (Figure 2A) and feature annotation using SCiLS™ Lab, iprm-PASEF enabled parallel MS/MS of up to 15 sulfatide precursor ions simultaneously (Figure 2B). In total, 15 region-focused iprm-PASEF runs provided full coverage of all sulfatide species present. Adjustment of the timsTOF fleX nitrogen gas pressure from 2.68 (default) to 2.10 mbar was performed after eight runs on SM4 and SM3 species in the *m/z* range of 500-1100. This enabled accurate determination of collisional cross sections for the multiglycosylated sulfatide species SM2a, SM1a, SM1b, and SB1a in the *m/z* range of 1200-1600, which could not be covered under default TIMS pressure settings [4]. Furthermore, a long ramp time of 480 ms enabled the direct separation of even- and odd-chain sulfatides on-tissue.

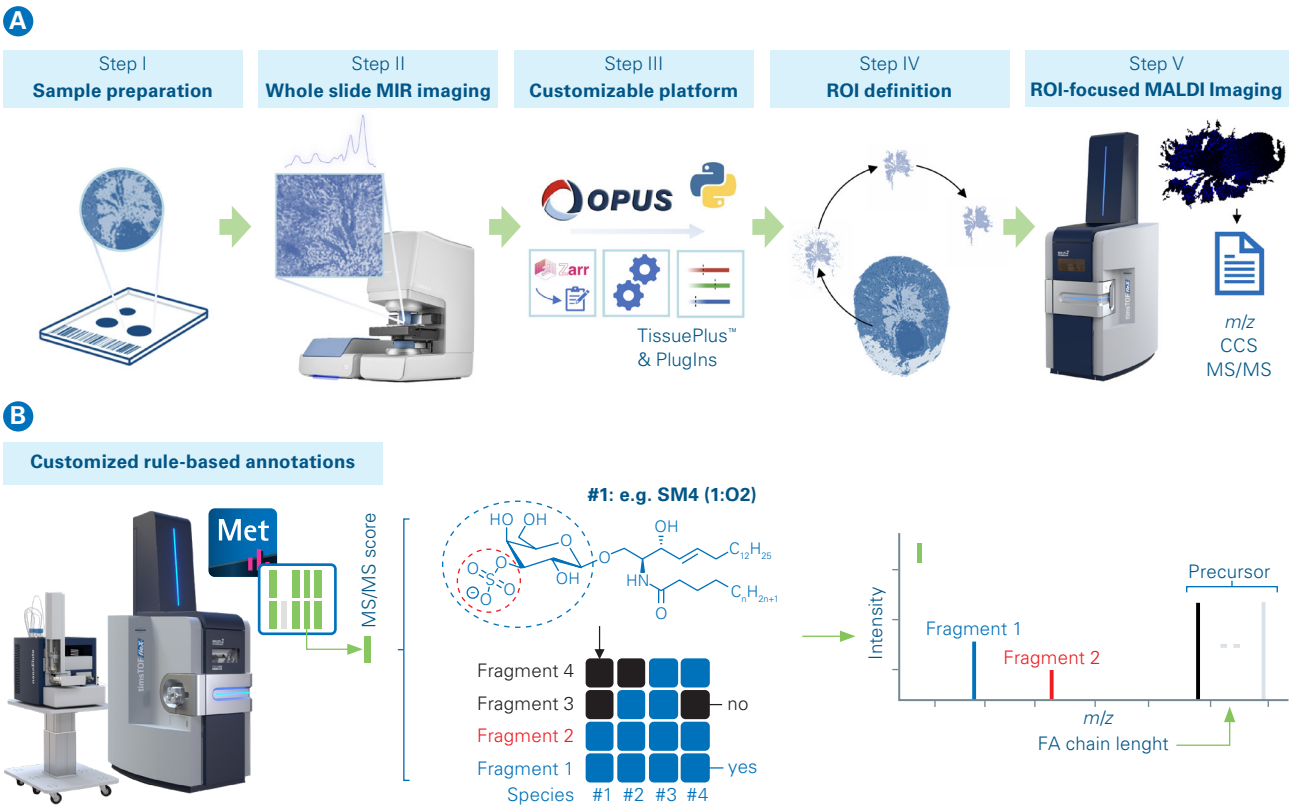

Figure 1. **Workflow overview QCL-MIR guided MALDI Imaging with iprm-PASEF of SM4 sulfatide species and customized rule-based annotations in MetaboScape.**
Ⓐ Schematic of the QCL-MIR imaging workflow for spatially focused data acquisition in MALDI Imaging from sample preparation to region-focused Imaging leading to a sulfatide database comprising CCS, exact mass and MS/MS fragment data [4]. Ⓑ Principle for customized rule-based annotations (MS/MS score) in MetaboScape exemplified on SM4 (1:O2) sulfatide. For lipid identification of a tissue extract, a customized rule-based annotation file was used based on the data generated in [4].

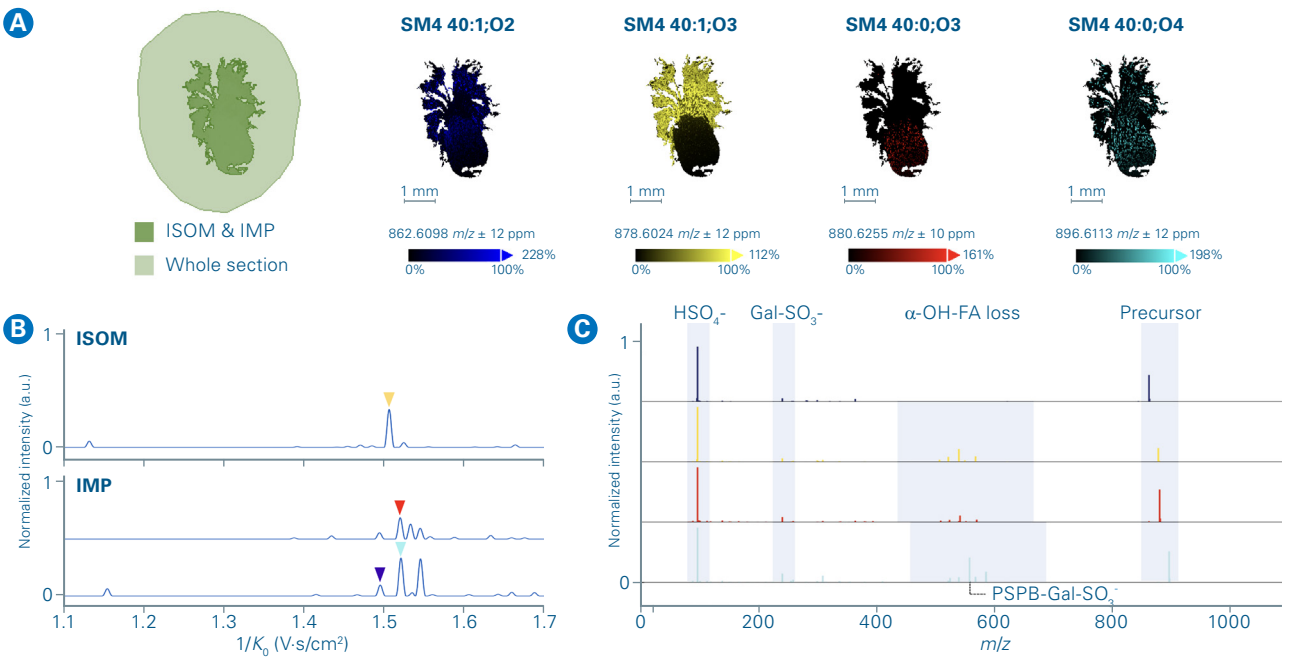

Figure 2. **QCL-MIR guided MALDI Imaging with iprm-PASEF of SM4 sulfatide species.**
Ⓐ Region-focused ion images of the kidney's ISOM and IMP. Ⓑ Baseline separated extracted ion mobilograms (EIM) containing the precursor ions of sulfatide species presented in Ⓐ. Ⓒ iprm-PASEF-derived MS2 spectra highlighting characteristic fragmentation patterns. The fragmentation pattern follows well-defined rules as elaborated in-depth for sulfatide species in [4].

Results

Structure-CCS-relationships and 3D hyperplane

The comprehensive foundation of QCL-MIR guided TIMS Imaging data for the given sulfatide classes enables structural investigations of CCS relationships across variable chain lengths, degrees of saturation, and headgroup diversity, the latter being described in detail in [4]. Structural trends for experimentally deduced CCS values of SM4(X:O2) species are depicted in Figure 3A. A linear model fit is used to discriminate the contribution of the fatty acid chain length (α) and the degree of saturation (β) on the CCS values. Series containing up to three double-bond equivalents (DBEs), SM4 42:X;O2 and SM4 44:X;O2, respectively, are fitted. Results for the structure-CCS-relationships of the headgroup diversity are given in [4]. In addition to the condensed 2D data visualization, the hyperplane regression model in Figure 3B provides an intuitive visual representation of the structural relationships, enabling CCS assignment for species lacking direct measurements and thereby further enhancing annotation confidence within the TIMS dimension. Furthermore, the sulfatide library improves the accuracy for CCS prediction tools built in MetaboScape, thus enabling hyperplane prediction even in the absence of sufficient reference data, e.g., for low-abundant sulfatide species detected in [4].

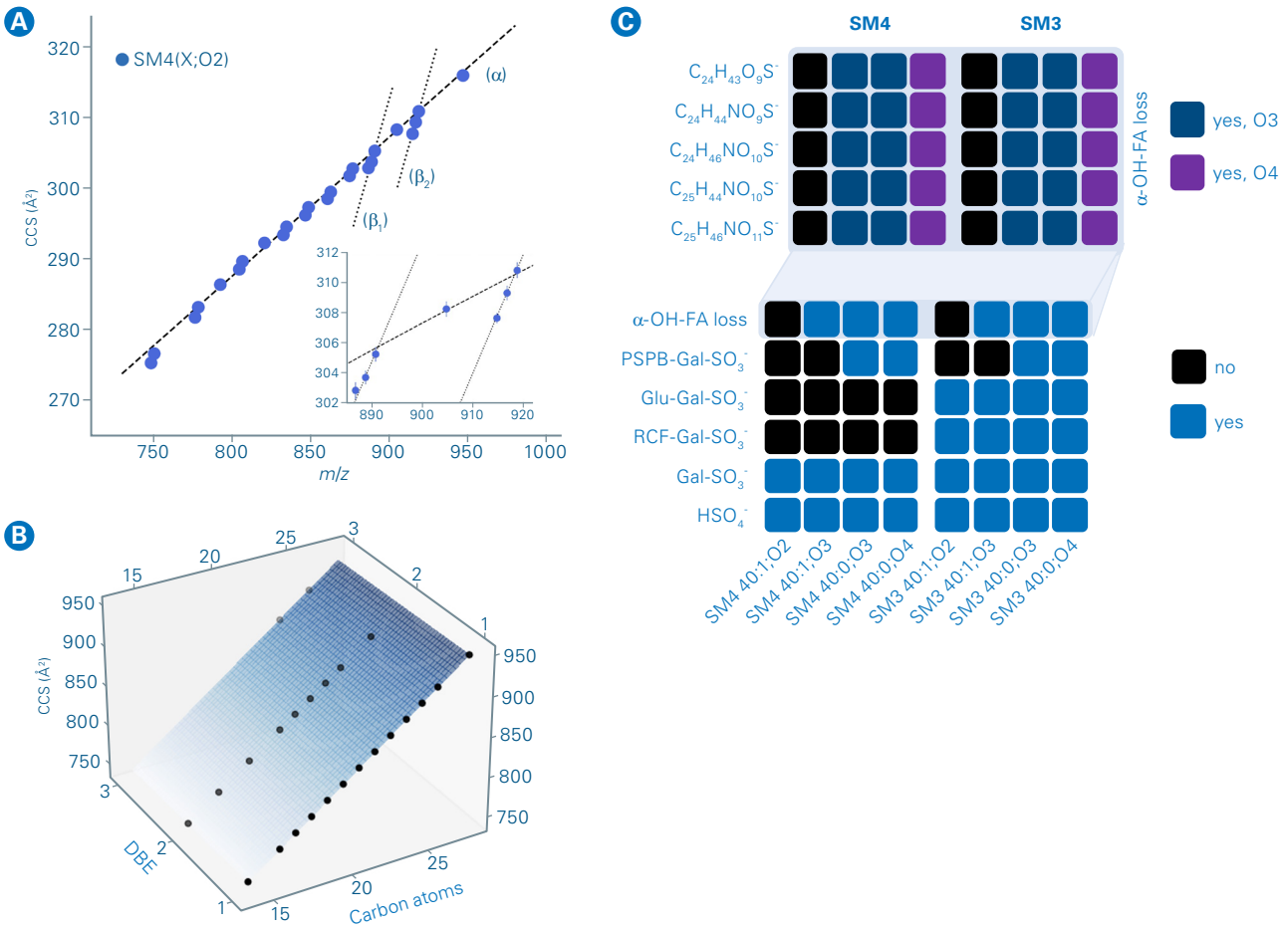

Figure 3. Deciphering sulfatide structural properties enables structure-CCS-relationship analysis, 3D regression modeling, and definition of well-defined fragmentation rules.

(A) Experimental CCS values for SM4(X;O2) and linear model fit as a function of their *m/z* value, evaluating the contribution of the fatty acid chain length (α) and the degree of saturation (β1 and β2). (B) The hyperplane provides a three-dimensional visual representation of the data presented in (A) for the given sulfatide subclass. Experimental values are depicted as black dots. (C) MS2 fragment patterns observed in iprm-PASEF analysis of selected SM4 and SM3 sulfatides. Fragment ions present in the MS2 spectra are indicated in blue. The α-hydroxy fatty acid loss is indicated by 5 correlated fragment ions. For O4 species, the indicated fragment ions further include H_2O and are therefore indicated as purple.

Rule-based sulfatide annotations

The evaluation of iprm-PASEF-derived MS2 spectra for 153 sulfatide species yielded 120 non-chimeric and 33 chimeric spectra, respectively. Subsequent detailed analysis of the fragmentation patterns across all sulfatides revealed recurring fragmentation patterns for the different subclasses (Figure 3C). Hereby, the most abundant fragment ions, including e.g., the hydrogensulfate ion, are considered. Understanding distinct fragmentation patterns enables the formulation of precise rules for MS2-based lipid annotation. In contrast to conventional similarity metrics, which risk outweighing individual features, rule-based annotation offers a robust and reproducible approach for accurate fragment ion assignment and lipid species identification. To enable rule-based lipid annotations across a broad range of molecular species, a preliminary (alpha) version of MetaboScape 2026b introduces the functionality for importing customized libraries for rule-based annotations. Based on the definition of fragment ions for the SM4 (X;O2) species presented in Figure 3A, a custom .json file employing the Mass Spec Query Language (MassQL[8]) was generated. For more information on how to create such a .json file, please contact your local sales representative (ms.sales.bdal@bruker.com). The MetaboScape rule-based annotation tool was then utilized to annotate lipid data from a tissue extract from ARSA$^{-/-}$ mouse kidney acquired using an Elute LC system coupled to a timsTOF Pro 2 instrument. Figure 4 illustrates a representative spectrum from SM4 40:1;O2, highlighting characteristic fragment ions corresponding to the sulfate and galactosyl-sulfate groups described in Figure 3C. The deconvoluted MS2 data enabled accurate and confident lipid annotation at the appropriate structural level.

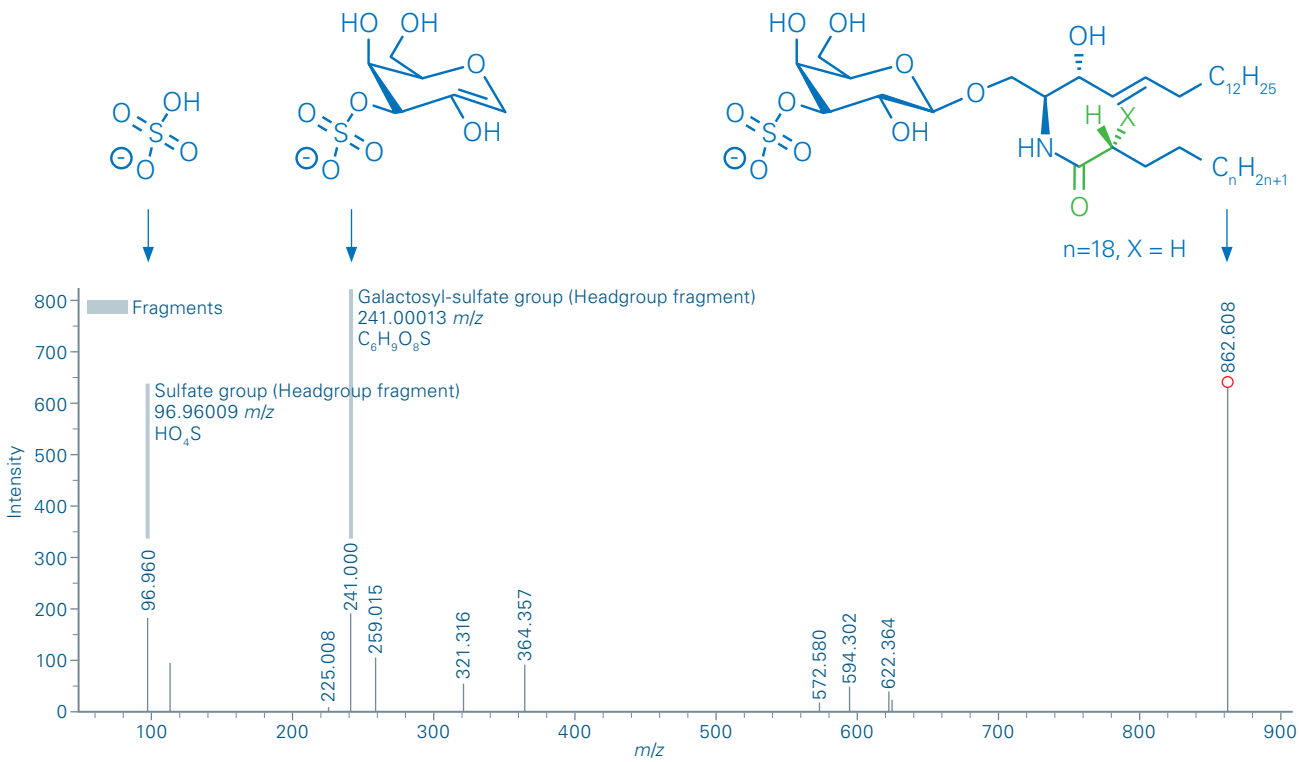

Figure 4. Rule-based lipid annotation in MetaboScape enables automated assignment of fragment ions in spatial lipidomics research. MS2 spectrum of SM4 40:1;O2 (*m/z* 862.61 [M-H]$^-$, $C_{46}H_{89}NO_{11}S$) including automatic assignment of the two characteristic fragments associated to the sulfate group (*m/z* 96.96 [M-H]$^-$, HSO_4^-) and the galactosyl-sulfate group (*m/z* 241.00 [M-H]$^-$, $C_6H_9O_8S^-$). The relative deviation of the measured CCS value to the fitted values from the hyperplane is 0.1%.

Conclusion

- QCL-MIR guided MALDI Imaging combined with iprm-PASEF enables a region-focused deep lipidomics workflow, supporting applications from single-cell analysis to 3D cell cultures and functional tissue areas in tissue.
- TIMS increases annotation confidence based on structure-CCS-relationships and CCS hyperplane models built from experimental reference data or predicted values.
- Automated rule-based lipid annotations in MetaboScape, using selected fragmentation rules for lipid subclasses, offer a streamlined solution for interpreting lipidomics datasets, as demonstrated for sulfatide species.
- The seamless acquisition-and-annotation workflow is also applicable to multimodal MALDI Imaging and LC-TIMS-MS implementations on the timsTOF fleX for in-depth spatial multiomics.

Be the first to know.

Subscribe here to receive updates on the next MetaboScape release and alerts about new features as soon as they go live.

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