GENProtocols

Protocol

Achieving Reproducible and Automated Region-Specific Lipid Annotations using CCS-aware SpatialOMx®

OMICS COMPUTATIONAL BIOSCIENCES

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Jul 27, 2021

Abstract

The CCS-aware SpatialOMx® workflow opens new dimensions by combining the molecular and spatial information measured by MALDI-TIMS Imaging with highly confident 4D-Omics annotations. MetaboScape® 2021b and SCiLS™ Lab 2021a provide the interface to match data from both ionization techniques and enable automatic and CCS-aware annotations of MALDI Imaging data. The CCS-value is a key component of this workflow.

Introduction

The in-depth analysis of lipids based on MALDI Mass Spectrometry imaging (MALDI Imaging) is still a challenging task. The main reason being the limited and nonautomated MS/MS capabilities in MALDI Imaging acquisitions. A solution to overcome this limitation is to run a 4D-Lipidomics™ annotation workflow in parallel to the imaging acquisition. The annotation results can be projected onto the spatially resolved molecular images.

The timsTOF fleX is especially well suited for this SpatialOMx® workflow as both ionization types are available on the same instrument and hence both approaches benefit from the mobility separation. In addition, parallel accumulation serial fragmentation, the PASEF® acquisition mode of the timsTOF fleX enables the 4D-Lipidomics™ workflow which combines sensitive lipidomics analysis with fast MS/MS acquisition rates [1].

The SpatialOMx® workflow is outlined in Figure 1. Its central feature is the matching of Omics-based annotations with the MALDI Imaging data. Besides the exact mass and the isotopic pattern information, MALDI trapped ion mobility spectrometry

(MALDI-TIMS) imaging provides the collisional cross section (CCS) value as an additional parameter for this inter-data matching. The TIMS device separates ions due to their different shapes in a gas flow giving access to the CCS-values. Furthermore, the additional orthogonal mobility separation increases the theoretical peak capacity of LC-MS analyses and thereby the depth of the analysis [2].

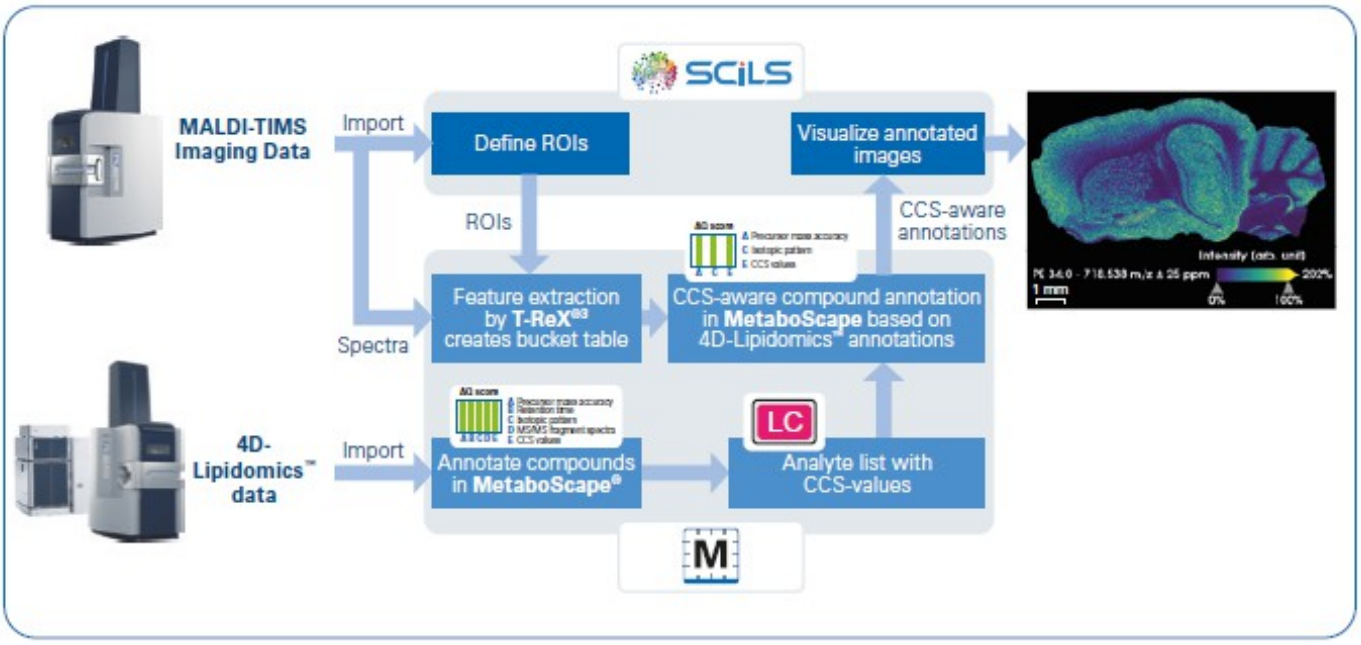


Figure 1: Schematic overview of the CCS-aware SpatialOMx® workflow for lipids. Features are extracted from 4D-Lipidomics™ data, annotated in MetaboScape® and exported as an Analyte List with CCS-values. MALDI Imaging data is imported to SCiLS™ Lab, where regions of interest (ROIs) are created. Spectral 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.

In comparison to other ion mobility technologies, TIMS features fast PASEF®-MS/MS acquisitions with high mobility resolving power and a duty cycle of up to 100% [3].

Here, we present the CCS-aware SpatialOMx® workflow and the mobility separation of isobaric lipid species on data from mouse brain samples. Both types of data (MALDI-TIMS Imaging and LC-PASEF®-MS/MS) were acquired on a timsTOF fleX mass spectrometer. The data fusion was done with MetaboScape® 2021b and SCiLS™ Lab 2021a.

Reagents and Equipment

MALDI-Guided SpatialOMx®

Procedure

The CCS-aware SpatialOMx® workflow streamlines the possibility to align features from different data types and applications with each other. Furthermore, MALDI-TIMS adds an additional dimension to MALDI images by adding the mobility separation.

A brain of a C57BL/6 mouse was divided into its two hemispheres with a sagittal cut. One half of the brain was snap frozen in liquid N2 for MALDI Imaging, while the other half was homogenized, and the lipids extracted according to a previously reported procedure [4].

The mouse brain lipid extract was analyzed using PASEF® LC-MS/MS mode on a timsTOF fleX instrument. Five injections were

measured for each polarity (see table 1 for parameters).

MS settings	timsTOF fleX
Source	Apollo II ESI source
Ionization	ESI(+), 4500 V Capillary Voltage ESI(-), 4200 V Capillary Voltage
Scan range	100–1500 *m/z*
Mobility range 1/K_0	0.55 – 1.90 V*s/cm²
Calibration	Internal mass calibration, Sodium Formate. Mobility calibration before sequence using Tunemix (Agilent, Santa Clara, USA)
PASEF	Positive mode precursors were fragmented from 300-1500 *m/z*. Negative mode precursors were fragmented from 100-1500 *m/z*.

UHPLC settings	Bruker Elute
Injection volume	2 µL (pos. mode), 10 µL (neg. mode)
Column	Bruker intensity C18 column (100 x 2.1 mm, 1.9 µm)
Column Oven Temp.	55°C
Flow Rate	0.4 mL/min
Mobile phase	A: acetonitrile / water (60:40, 10 mM NH_4Formate, 0.1% FA) B: isopropanol / acetonitrile (90:10, 10 mM NH_4Formate, 0.1% FA)
Gradient	0 min 40% B 2 min 43% B 2.1 min 50% B 12 min 54% B 12.1 min 70% B 18 min 99% B 18.1 min 40% B 20 min 40% B
Data processing and evaluation	MetaboScape® 2021b

Table 1: UHPLC MS setup for lipid profiling

The raw data were processed with MetaboScape® 2021b, using four-dimensional feature extraction (T-ReX® 4D). All qualifiers (exact mass, isotopic pattern, retention times, MS/MS spectra and CCS-values) were extracted automatically for all adducts. The retention time aligned features were listed in a bucket table. The data was annotated using a new rule-based lipid annotation tool that is integrated in MetaboScape® 2021b. It uses published fragmentation rules based on [M+H]+, [M+Na]+, [M+NH4]+, [M-H]-, [M+HCOO]- and [M+CH3COO]- ions from currently 41 sub-classes out of four main categories (Glycerolipids, Glycerophospholipids, Sphingolipids and Sterol lipids). The syntax of the rule-based annotation in MetaboScape® 2021b is following the annotation concept presented by Liebisch et al. [5], which is recommended by the Lipidomics Standards Initiative (LSI) [https://lipidomics-standards-initiative.org/] and supports identifications on the species or the molecular species level.

The plausibility of these annotations was rated by the Annotation Quality (AQ) scoring. An "Analyte List" was generated from all annotated lipids of the positive/negative mode merged data. This list was used for annotation of the MALDI data. The

matching between MALDI and ESI data was based on mass accuracy. The CCS-value served as an additional validation criterion. Annotations were considered only if the respective CCS deviations between MALDI- and ESI data were below 2%.

For MALDI Imaging, the corresponding fresh frozen mouse brain was sectioned at 10 µm thickness and mounted onto conductive glass slides (Bruker Daltonik GmbH, Bremen, Germany). After drying, sections were sprayed with 2.5 mg/mL ZSA matrix in 70% ACN/H2O using a TM sprayer (HTX Technologies, Chapel Hill, NC, USA) [6]. Tissues were measured in negative ion mode with 20 or 50 µm pixel size in the mass range 300-1200 m/z. The smartbeam 3D laser was operated at 10 kHz repetition rate. 400 shots per pixel were collected with a single beam scan laser pattern. MALDI-TIMS Imaging data were acquired with 800 ms ramp time in the 1/K0 range from 0.6-1.8 V·s/ cm2.

The MALDI Imaging data were imported into SCiLS™ Lab 2021a software. Regions of interest (ROIs) were deduced from an unsupervised segmentation map which was calculated in SCiLS™ Lab 2021a. Spectral- as well as ROI-information from five segments were imported to MetaboScape® 2021b for automatic lipid annotation using the Analyte List previously created in the 4D-Lipidomics™ analysis.

Results

4D-Lipidomics™ yields highly confident lipid annotations from ESI data

292 unique lipid annotations were obtained from the 4D-ESI data in negative ionization mode and 323 from positive mode data. 446 lipids were annotated in the Bucket table merged by MetaboScape®. An Analyte List was compiled from the merged bucket table and used for the subsequent CCS-aware matching of the MALDI Imaging data. An overview on the lipid classes assigned in the ESI data is shown in Figure 2. A color code visualizes in which class an annotated lipid belongs. Lipids of the same class are expected to cluster together due to the utilized reversed phase chromatography. This allows to easily see potentially wrong annotations. MetaboScape® 2021b allows further validation by e.g. using 4D Kendrick Mass Defect (KMD) plots, validation of structure candidates using in-silico fragmentation [7] or the comparison with predicted CCS-values (CCSPredict).

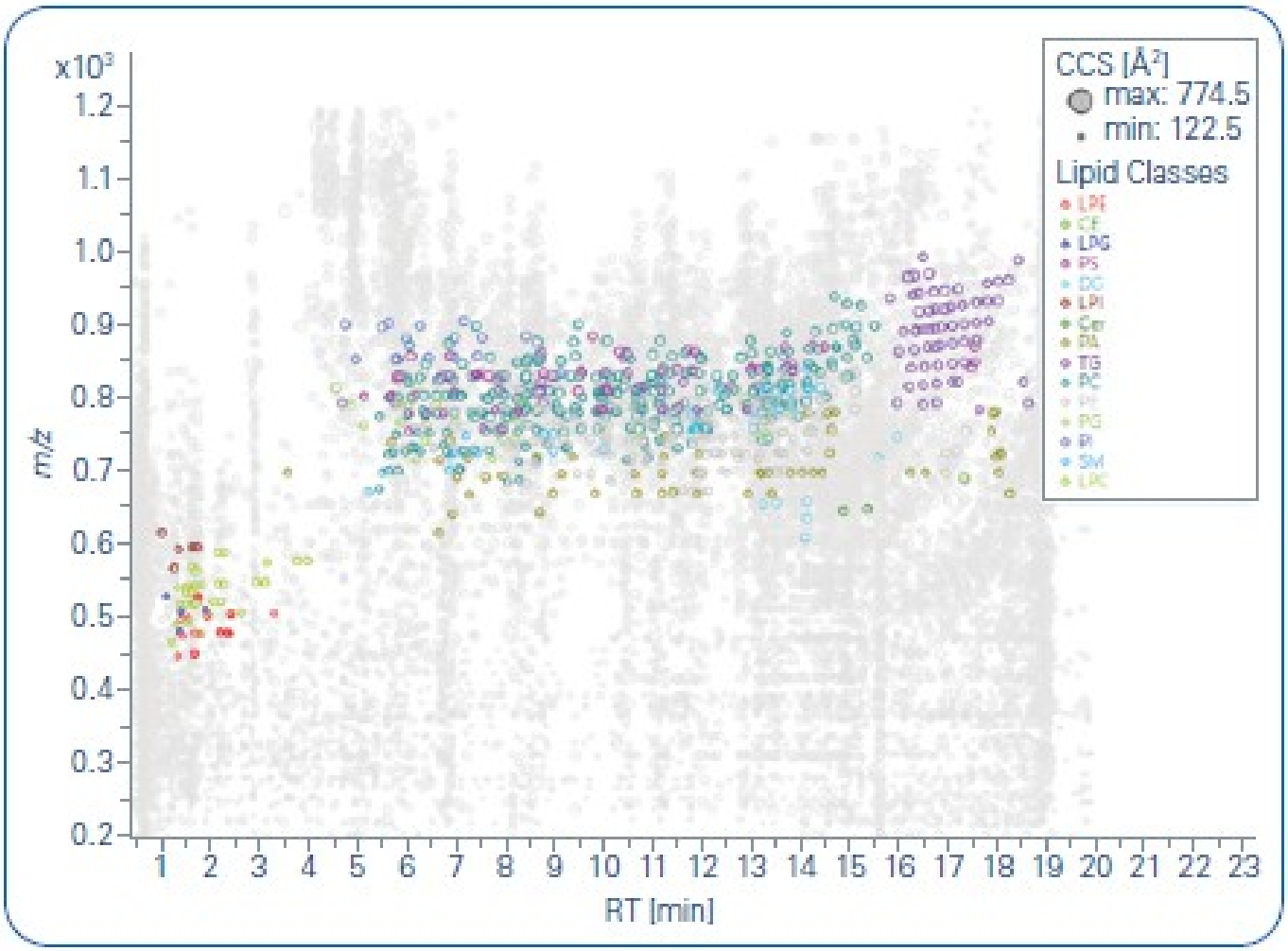


Figure 2: Lipid classes annotated by the rule-based lipid annotation tool.

The KMD plot for PC and lyso PC lipids is shown in Figure 3. The retention time is visualized by color coding and the CCS values are represented by the bubble size. LPCs show consistently smaller CCS than PCs. Potential outliers, e.g. showing much larger retention times could easily be detected.

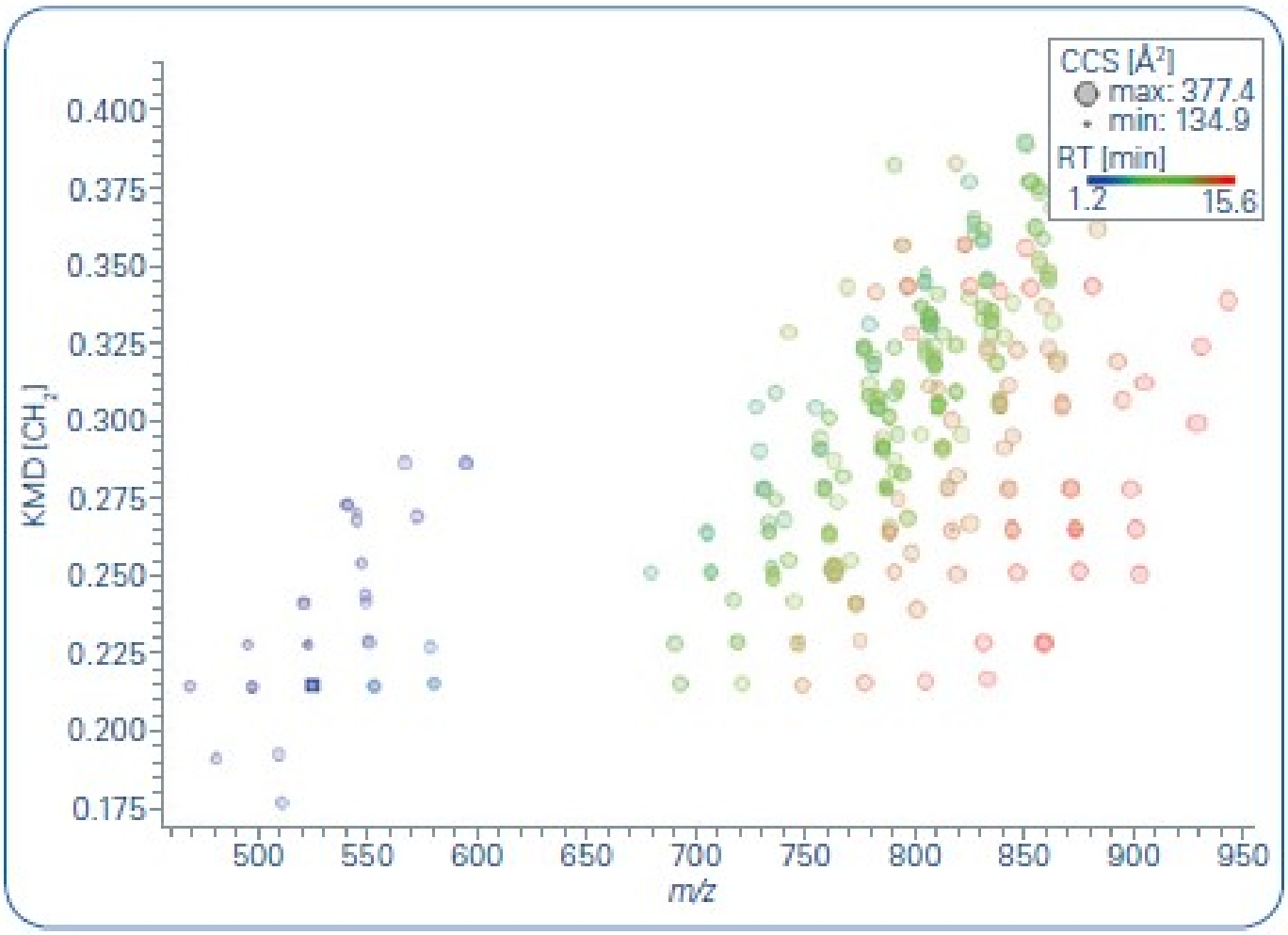


Figure 3: Kendrick Mass Defect (KMD) plot of PC (>650 m/z) and lyso PC (<600 m/z) lipids. This view can be used to screen for potential false positive annotations or further non-annotated candidates of a series.

The KMD plot can also be used to analyze series or families with either similar KMDs (horizontal lines, Figure 4a) or as diagonal lines when lipids had the same chain length but a different degree of saturation (Figure 4b). Some annotations show sufficient fragment information to annotate on a molecular species level.

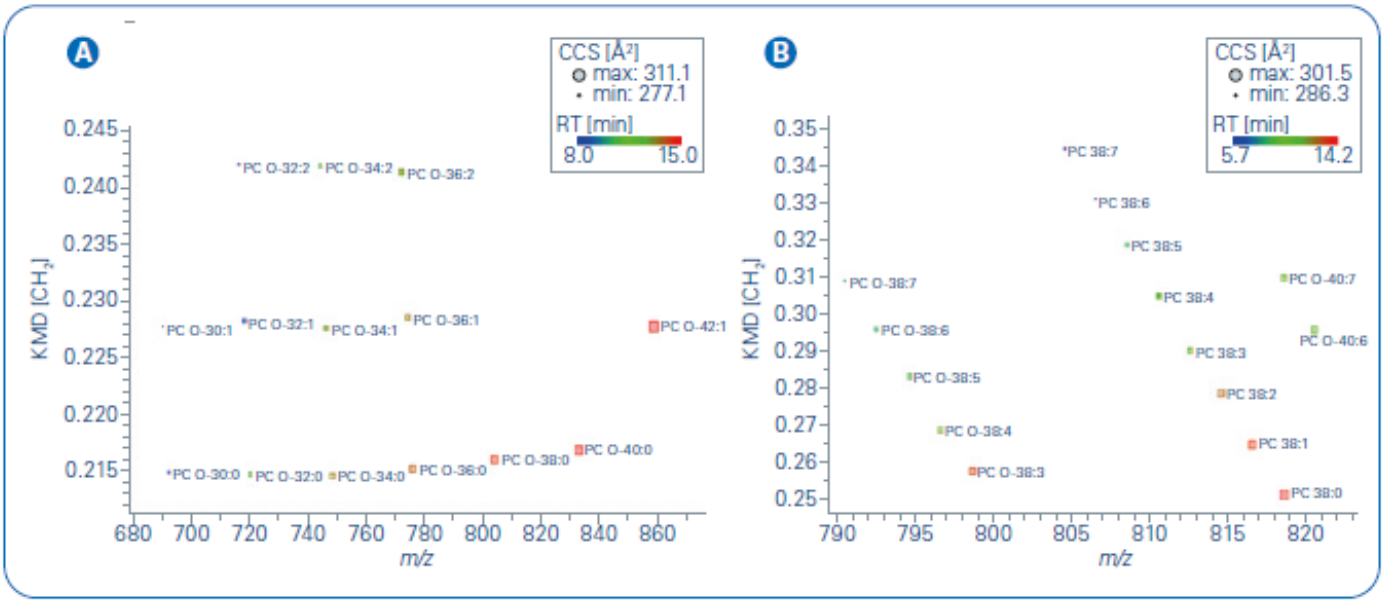


Figure 4: Lipids with similar degree of saturation form horizontal series in the KMD plot A, while lipids with different numbers of double bonds but same chain length line up diagonally B.

CCS-values are reproducible across ionization methods

CCS-aware annotation brings an additional criterion for the matching of MALDI Imaging and LC-ESI-PASEF® data. This requires reproducible CCS-values, not only from measurement to measurement but also across ionization techniques. Figure 5 depicts such a comparison between technologies. The maximum variation of CCS for the presented lipids is in the range of 1%. This proves the validity of the SpatialOMx® approach and demonstrates the benefit of having CCS as an additional matching criterion.

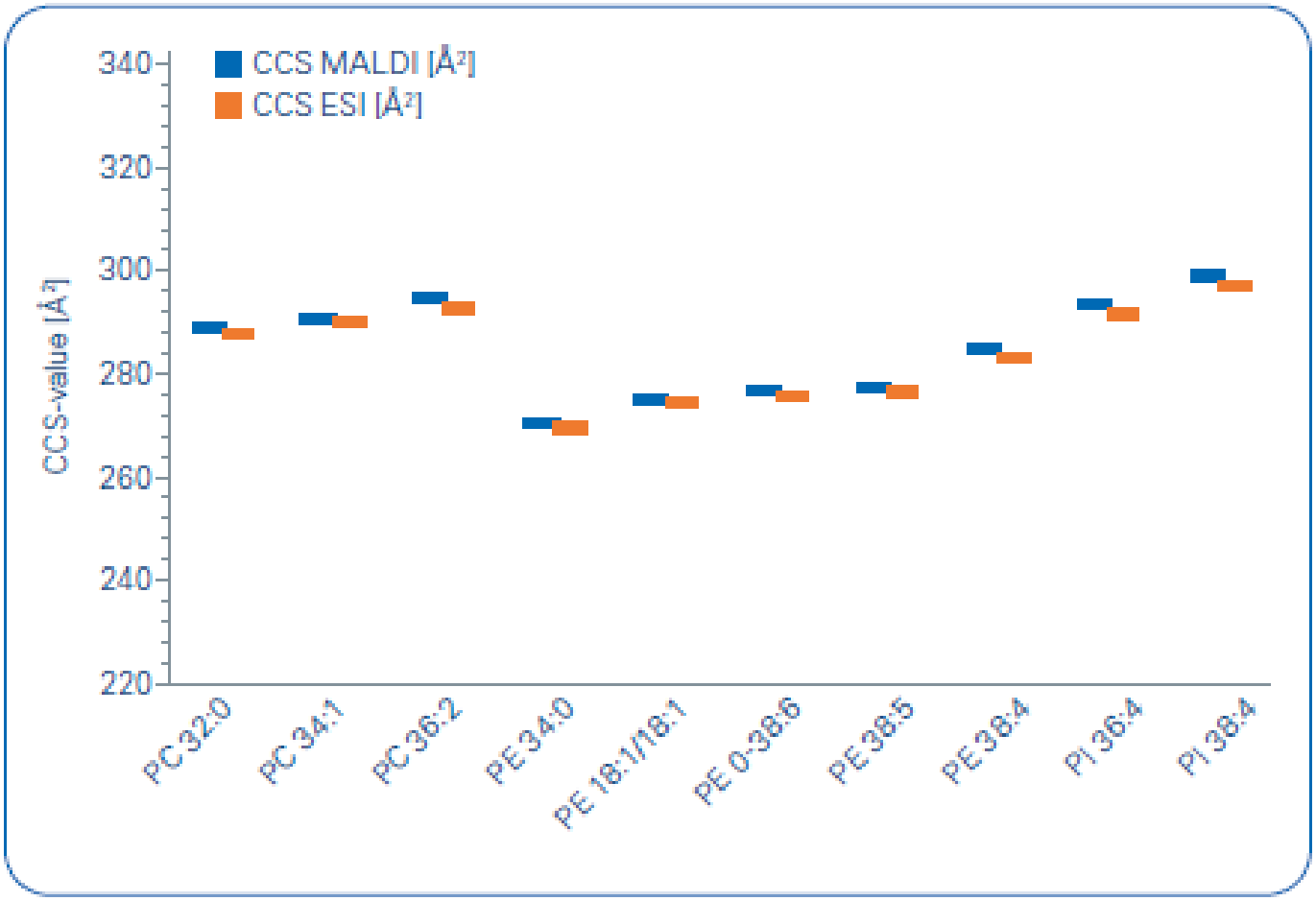


Figure 5: Reproducibility of CCS-values across ESI- and MALDI-ionization for different lipids.

CCS-aware annotation of MALDI Imaging data

The unsupervised segmentation tool in SCiLS™ Lab creates a segmentation map in which "similar pixel" (meaning similar MALDI spectra in this context) are grouped to segments sharing the same color. These segments can be regions with the same or very similar phenotype. The transferability of such MALDI-based images to microscopy images of stained samples is well known [8]. Five regions of interest (ROIs) were deduced from the automatically generated map of the negative mode MALDI Imaging data. Figure 6 shows the segmentation map and the respective regions of interest. The region annotation can be assigned by the user in SCiLS™ Lab. The main anatomical regions of the mouse brain, such as e.g. white matter, granular layer and molecular layer of the cerebellum, corpus callosum and cortex were reflected by the segmentation map.

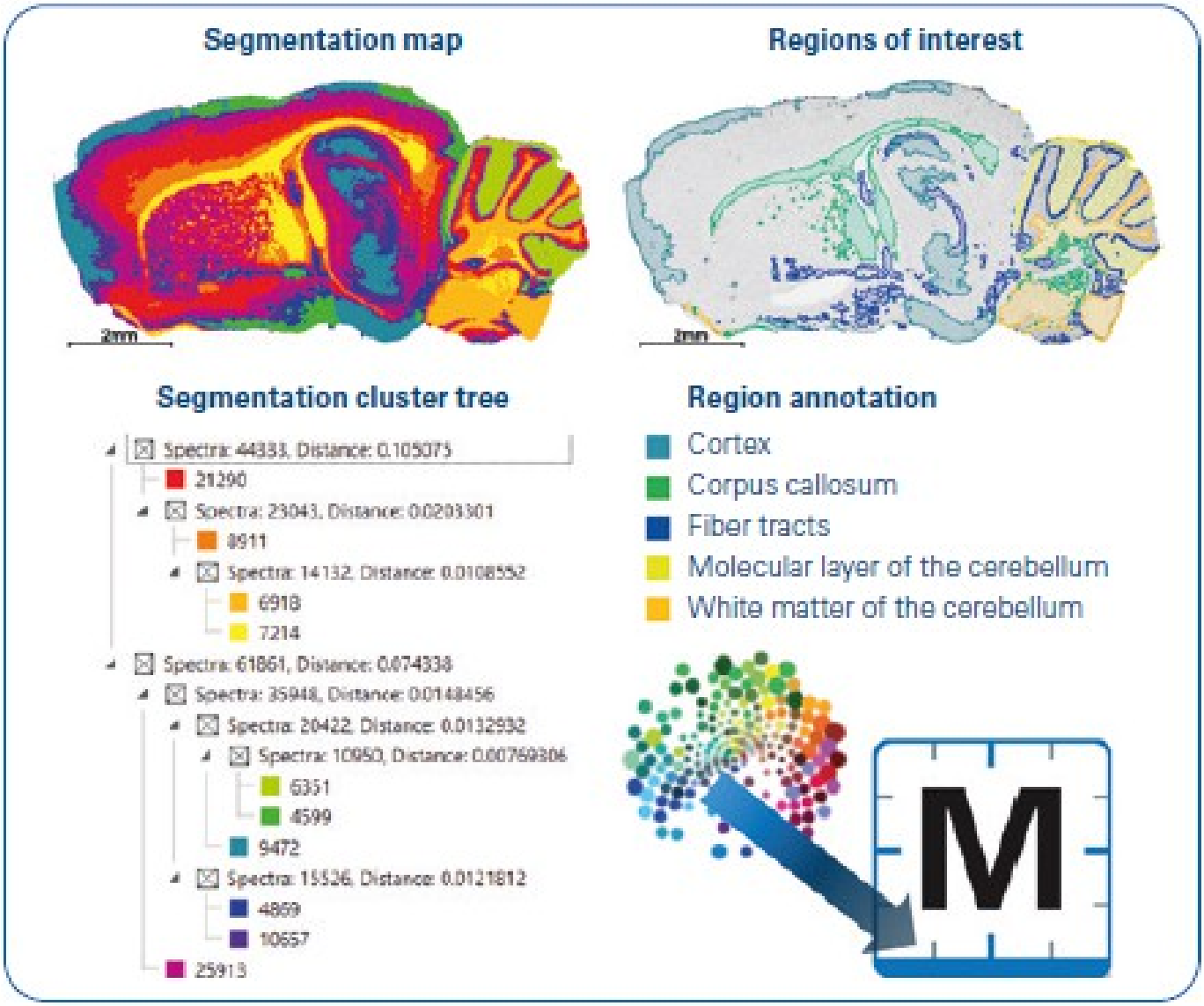
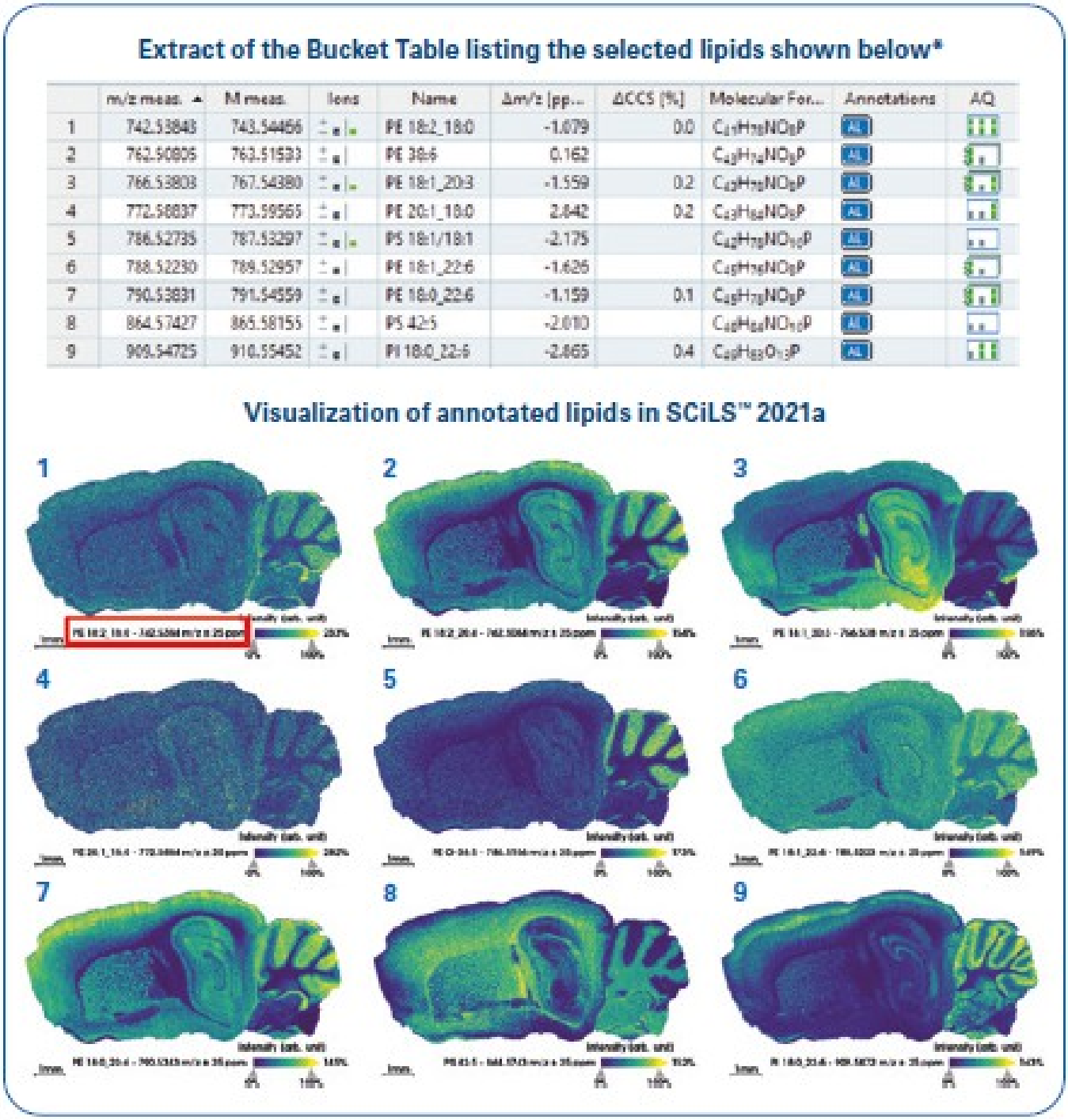


Figure 6: SCiLS™ segments (left hand side) are converted to regions of interest (right hand side). These can be exported for further processing to MetaboScape® 2021.

Imported into MetaboScape® 2021b, the T-Rex®3 feature extraction algorithm performs a de-isotoping and de-adducting of MALDI data. The resulting bucket table was annotated by matching against the 4D-Lipidomics™ Analyte List that was created from the ESI data. For matching, the exact mass and CCS-value were used. 82 lipids (20% of the total number) were detected in both data types. The list of annotated lipids was finally imported to SCiLS™ Lab for visualization.

Figure 7 shows one of the two benefits from the SpatialOMx® workflow exemplary for nine lipids: While previously MALDI images could only be labelled with the m/z values, now the MS/MS verified lipid annotations can be used.



	m/z meas.	M meas.	Ions	Name	Δm/z [pp...	ΔCCS [%]	Molecular For...	Annotations	AQ
1	742.53843	743.54466		PE 18:2_18:0	-1.079	0.0	$C_{41}H_{78}NO_8P$		
2	762.50805	763.51533		PE 38:6	0.162		$C_{43}H_{74}NO_8P$		
3	766.53803	767.54380		PE 18:1_20:3	-1.559	0.2	$C_{43}H_{78}NO_8P$		
4	772.56837	773.59565		PE 20:1_18:0	2.842	0.2	$C_{43}H_{86}NO_8P$		
5	786.52735	787.53297		PS 18:1/18:1	-2.175		$C_{42}H_{78}NO_{10}P$		
6	788.52230	789.52957		PE 18:1_22:6	-1.626		$C_{45}H_{76}NO_8P$		
7	790.53831	791.54559		PE 18:0_22:6	-1.159	0.1	$C_{45}H_{78}NO_8P$		
8	864.57427	865.58155		PS 42:5	-2.010		$C_{48}H_{84}NO_{10}P$		
9	909.54725	910.55452		PI 18:0_22:6	-2.865	0.4	$C_{49}H_{83}O_{13}P$		

Figure 7: Selected lipid annotations in the MetaboScape® 2021 bucket table (top) and their spatial distribution visualized in SCiLS™ Lab (bottom). Note that SCiLS™ depicts in addition to the measured m/z-value also the lipid annotation (see the red box). *Missing deltaCCS indicates that a different adduct was detected with ESI.

Processing of MALDI-TIMS Imaging data in SCiLS™ Lab 2021a

The second unique feature of the CCS-aware SpatialOMx® workflow is presented in Figure 8: SCiLS™ Lab 2021a visualizes mass- and mobility pair images.

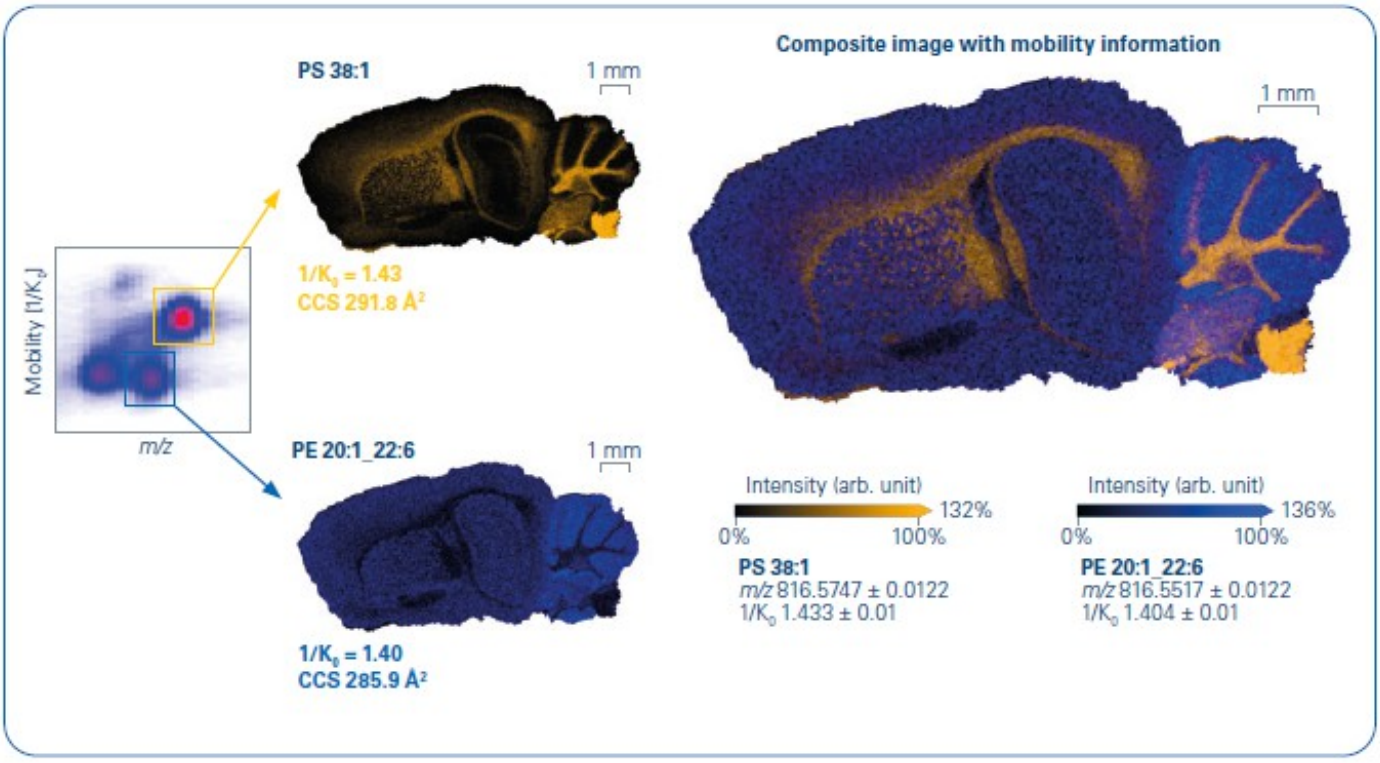


Figure 8: Visualization of MALDI-TIMS Imaging data in SCiLS™ Lab 2021a.

To demonstrate the benefit of mobility separation, two isobaric compounds with a neutral nominal mass of 816 were selected. These compounds were annotated as PE 20:1_22:6 and PS 38:1 in the 4D-Lipidomics™ data. The annotation was supported by MS/MS fragment spectra information. The CCS value for the protonated species of PE_20:1_22:6 was determined as being 285.9 Å2 for both ionizations, providing additional matching confidence.

The two lipids deviated in the exact masses by only 23.7 mDa, but the additional TIMS separation was able to separate them. This helped to acquire clean MS/MS spectra using ESI and to visualize the spatial distribution for both lipids using MALDI.

SCiLS™ Lab 2021a features the extraction of the mass and mobility pair images and displays the corresponding images. Interestingly, the distribution of the two compounds varied. PE 20:1_22:6 was mainly localized in the gray matter of the cerebellum. In contrast, PS 38:1 was mainly localized in the white matter of the cerebellum, corpus callosum and spinal nucleus.

Only the additional mobility separation allowed the differentiation and sophisticated visualization as separate MALDI images. In summary, TIMS is especially helpful for the analysis of complex lipid samples. Including the spatial dimension by MALDI Imaging adds the tissue context to lipidomics data.

Conclusion

- Mobility enhanced MALDI-TIMS Imaging enables the separation of isobaric or even isomeric compounds and thereby delivers unprecedented imaging results, especially for spatial lipidomics.
- The novel CCS-aware SpatialOMx® workflow increases the confidence in lipid annotations for MALDI images through the acquisition of CCS-tagged data.
- The CCS-aware SpatialOMx® workflow is facilitated by a seamless communication between MetaboScape® 2021b and SCiLS™ Lab 2021a.

Notes and Comments

Full applications note.

References

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