



iprm-PASEF for on-tissue MALDI MS/MS Imaging

Introducing a new integrated workflow for more confidence in MS/MS-based molecular identification for MALDI Imaging data

Introduction

Spatial multiomics approaches are becoming more prevalent in probing molecular mechanisms underlying diseases and therapies. [MALDI Imaging](#) plays a pivotal role by uncovering spatial patterns across various omics layers within tissue samples. Its label-free nature of lipids, metabolites, glycans, and other small molecules necessitates complementary analyses for accurate identification.

While MS/MS-based methods are ideal for this, obtaining and annotating spatial MS/MS data at a reasonable throughput can still be a challenge. In this technical note, we introduce imaging parallel reaction monitoring (iprm) with parallel accumulation serial fragmentation (PASEF[®]) (iprm-PASEF[®]), an integrated software workflow for targeted, multiplexed MALDI MS/MS Imaging, enabling acquisition, analysis, and annotation of fragment ion images for up to 25 precursor ions in one run. Employed during a MALDI Imaging acquisition, iprm-PASEF allows for parallel MS/MS imaging of up to 25 mobility-resolved precursor isolation windows while maintaining the spatial context of both precursor and fragment ions.

iprm-PASEF takes advantage of the trapped ion mobility spectrometry (TIMS) technology in the [timsTOF fleX](#) to increase the multiplexing capability dramatically for MS/MS imaging. It combines the targeted precursor selection of parallel reaction monitoring (PRM) with PASEF to enable the mobility-resolved fragmentation of analytes that are sequentially eluting in the ion mobility dimension from the TIMS device into the collision cell of the timsTOF instrument.

Keywords:
MALDI Imaging,
timsTOF fleX,
MS/MS imaging,
TIMS, iprm-PASEF,
SCiLS Lab,
MetaboScape

SCiLS™ Lab supports the iprm-PASEF workflow by exporting the precursor data (m/z and $1/K_0$ range) from a preceding collisional cross section (CCS)-enabled MALDI Imaging run (termed MS or precursor run), which can include regions of interest (ROIs) for a guided MS/MS acquisition. In iprm-PASEF data sets, the T-ReX³ feature finding algorithm extracts mobility-resolved MS/MS spectra as it detects, groups, and links the features generated by the intact precursor and fragment ions. Using the MetaboScape®-powered molecular annotation workflow, which utilizes MS and MS/MS data, precursor molecules can be identified using the Lipid Species annotation and Target List with high confidence (Figure 1).

Methods

Materials and sample preparation

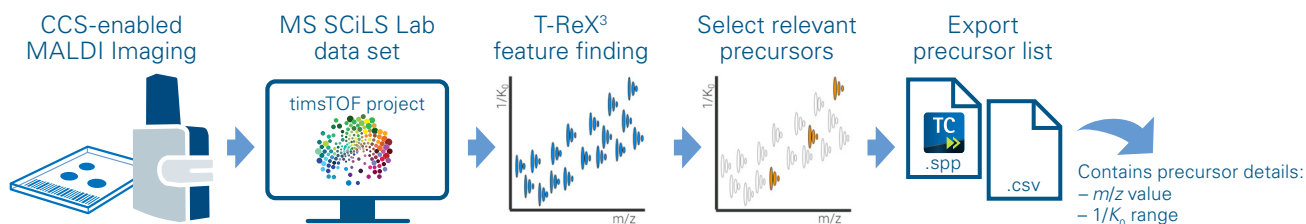
Rat brain cryosections were prepared at 10 μm thickness on Bruker IntelliSlides® and spray-coated with NEDC matrix using standard protocols on an HTX M3+ sprayer (Chapel Hill, NC).

Mass Spectrometry

MALDI Imaging measurements were performed on a timsTOF fleX instrument in negative ion polarity mode. The instrument was operated using timsControl 6.0 software with a default lipid imaging acquisition method covering m/z 300–1350. m/z calibration was performed using red phosphorus in MALDI mode, and $1/K_0$ calibration used tune mix in ESI mode by direct infusion.

The MALDI Imaging run was set up using flexImaging 7.5 software, with a pixel size of $20 \times 20 \mu\text{m}^2$ and a laser ablation area of $10 \times 10 \mu\text{m}^2$. This strategy allows for the independent acquisition of four $10 \times 10 \mu\text{m}^2$ quadrants in one $20 \times 20 \mu\text{m}^2$ pixel area (see Figure 2 for more details).

Step 1: Acquire CCS-enabled MS data to generate a list with target precursors in SCiLS Lab



Step 2: Acquire iprm-PASEF MS/MS data and annotate target precursors using MetaboScape-powered molecular annotation

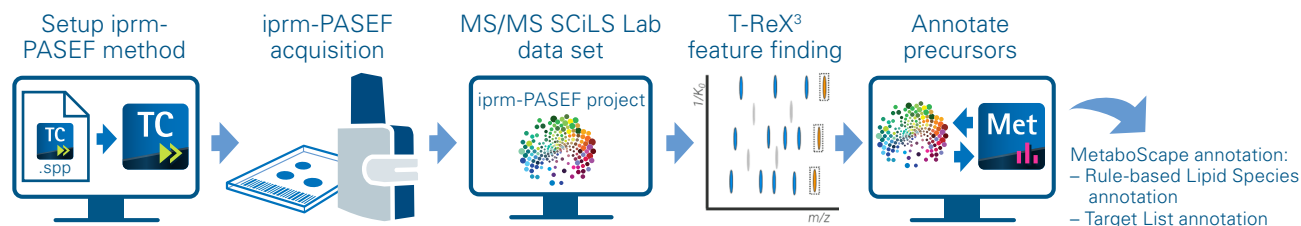


Figure 1

General workflow for iprm-PASEF acquisition and analysis.

The first step is the acquisition of a MS imaging data set, which is used for precursor selection. Then, these precursors' information, including the m/z and $1/K_0$ range, can be exported from SCiLS Lab to timsControl software to create an iprm-PASEF acquisition method. Following the iprm-PASEF acquisition, data is imported into an iprm-PASEF SCiLS Lab project, in which the T-ReX³ feature finding algorithm is used to generate MS/MS spectra. For the last step, these MS/MS spectra can be imported and annotated using the MetaboScape-powered molecular annotation workflow.

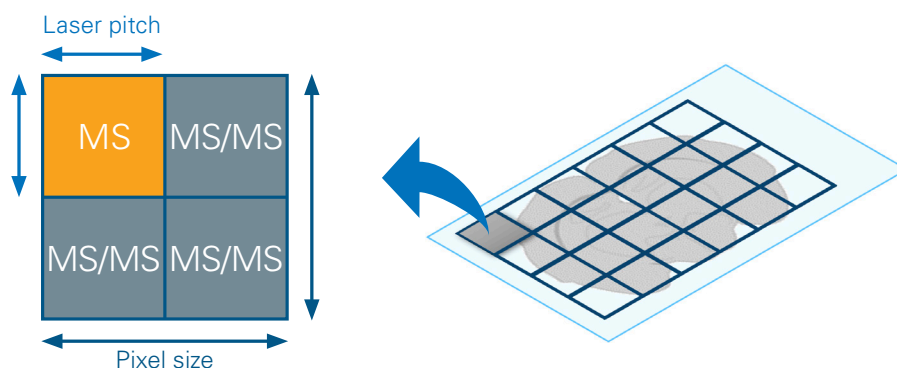


Figure 2. Pixel subsampling powered by the smartbeam 3D laser.

Selecting a laser pitch twice as large as the laser ablation area allows for dividing a MALDI pixel into four subpixels. This increases the number of possible independent imaging runs per sample, e.g., one MS run followed by three iprm-PASEF MS/MS runs.

For iprm-PASEF, the acquisition method was set to the prrm-PASEF Scan Mode, and tuning parameters were optimized to ensure proper ion transmissions for precursor and fragment ions. The main parameters to tune are the Collision Cell and Focus Pre TOF settings, which are essential devices for ion fragmentation. Details of the parameters that may need to be modified when transitioning from a MALDI TIMS MS method to an iprm-PASEF method are listed in Table 1.

Table 1. Differences in the tune parameters between the MALDI TIMS MS and the iprm-PASEF acquisition method.

Tune Parameter	TIMS MS	iprm-PASEF
<i>m/z</i> range	300-1350	50-1350
Collision Cell		
Collision Energy [eV]	10	25
Collision RF [Vpp]	1800	800
Focus Pre TOF		
Transfer Time [μ s]	85	75
Pre Pulse Storage [μ s]	10	10

Data processing

Both MALDI MS and iprm-PASEF MS/MS data sets were analyzed in SCiLS Lab 2025a, including precursor scheduling, and the Lipid Species annotation powered by MetaboScape 2025 software.

Results

Precursor selection from MALDI Imaging data

The iprm-PASEF workflow starts off with a previously recorded MS data set to define precursor ions of interest for subsequent MS/MS analysis. To obtain accurate m/z and $1/K_0$ values of all possible precursors, the T-ReX³ feature finding algorithm was employed in SCiLS Lab. Next, a feature filtering was done based on the PC1 loading values of a PCA analysis among the brain subregions. The largest variation (PC1) in the brain tissue was caused by the difference between white and grey matter, and as such, this filter resulted in a list of features with a higher abundance in the white matter of the rat brain. Other statistical tools (e.g., ROC analysis, colocalization, or correlation) in SCiLS Lab can also be used to filter features of interest. In this study, 12 features were selected as precursor candidates for the follow-up iprm-PASEF MS/MS experiment.

The molecular annotations of these 12 lipid precursors were conducted using the rule-based Lipid Species annotation tool from MetaboScape 2025 based on accurate m/z and CCS values. Lacking MS/MS information, the reported annotations are at the lipid species level (i.e., without detailed information on the fatty acyl composition) (Figure 3A). For instance, the feature at m/z 701.5124 was annotated as PA 36:1.

For iprm-PASEF precursor scheduling, the feature list was exported to timsControl via the "Export iprm-PASEF parameters" dialog (Figure 3B), including m/z value and non-overlapping $1/K_0$ start and end values. In this dialog, the active feature list was sorted by increasing $1/K_0$ values. Overlapping or narrowing of neighboring isolation windows ($1/K_0$ distance below 0.01 Vs/cm^2) need to be modified before continuing with the precursor list export, as no meaningful MS/MS spectra can be generated from such narrow isolation windows.

This can be achieved by first deselecting overlapping features as shown for m/z 528.3080, then either manual editing of the mobility window or using the auto-resolve option as shown for m/z 701.5124 and m/z 729.5402.

The auto-resolve option attempts to automatically adjust the mobility intervals of neighboring isolation windows while ensuring that the original mobility center value of the CCS features remains within the isolation window. If all precursor isolation windows are valid, the precursor list can be exported to either .csv or the SCiLS prm-PASEF parameter (.spp) file format. The latter exclusively contains region information to guide the iprm-PASEF acquisition to a certain (sub)-region of the original sample to reduce the total acquisition time. Here, only the white matter coordinates were exported for MS/MS acquisition, based on PCA results.

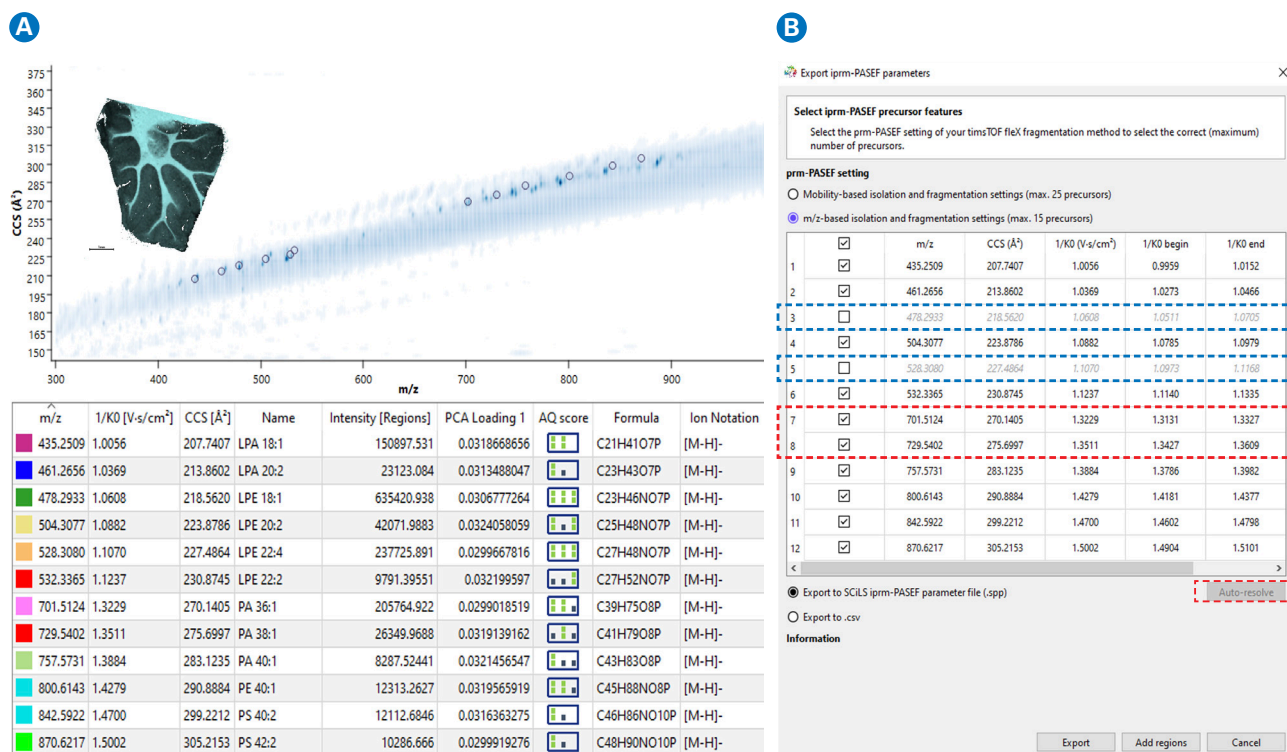


Figure 3. (A) SCiLS Lab feature navigator displaying 12 lipid features that were selected for iprm-PASEF export. (B) In the export iprm-PASEF parameters window, the feature list is sorted by the precursor's mobilities, including $1/K_0$ begin and end values. Precursor windows with overlapping boundaries were resolved by deselecting m/z 478.2933 and m/z 528.3080 (blue box). The precursor windows for m/z 701.5124 and m/z 729.5402 (red box) initially had a mobility gap below 0.1 Vs/cm^2 and were resolved by the "auto-resolve" function to ensure proper precursor isolation. The 10 remaining features were exported as .spp file including ROI coordinates.

iprm-PASEF acquisition

In timsControl software, the optimized prM-PASEF method was loaded, and the isolation and fragmentation settings were defined for the iprm-PASEF analysis of lipids (Figure 4A). In the prM-PASEF windows editor, the .spp file was imported to analyze the 10 precursors as defined in SCiLS Lab (Figure 4B). If needed, the isolation windows can be modified or added manually. In case of overlapping or too narrow mobility windows, which could compromise data quality, respective warnings are shown for user awareness in yellow or red. The applied m/z isolation width and collision energies for each window get interpolated based on the profile defined in the isolation and fragmentation settings. To review the validity of defined isolation windows, data from the previous MALDI MS run can be opened in the prM-PASEF window editor (Figure 4B). For imaging acquisition, the defined precursor windows need to be saved with the method.

After calibration of the mass and mobility dimension, the imaging run was created in flexImaging with the optimized iprm-PASEF method and the ROI information imported from the .spp file. This allows to exclusively analyze the white matter area as defined within SCiLS Lab. As the first quadrant of the $20 \times 20 \mu\text{m}^2$ pixel was used for the MALDI MS analysis, a $10 \mu\text{m}$ offset (in the x-dimension) was defined in the run options upon the start of the next iprm-PASEF acquisition (see Figure 2 for graphical representation).

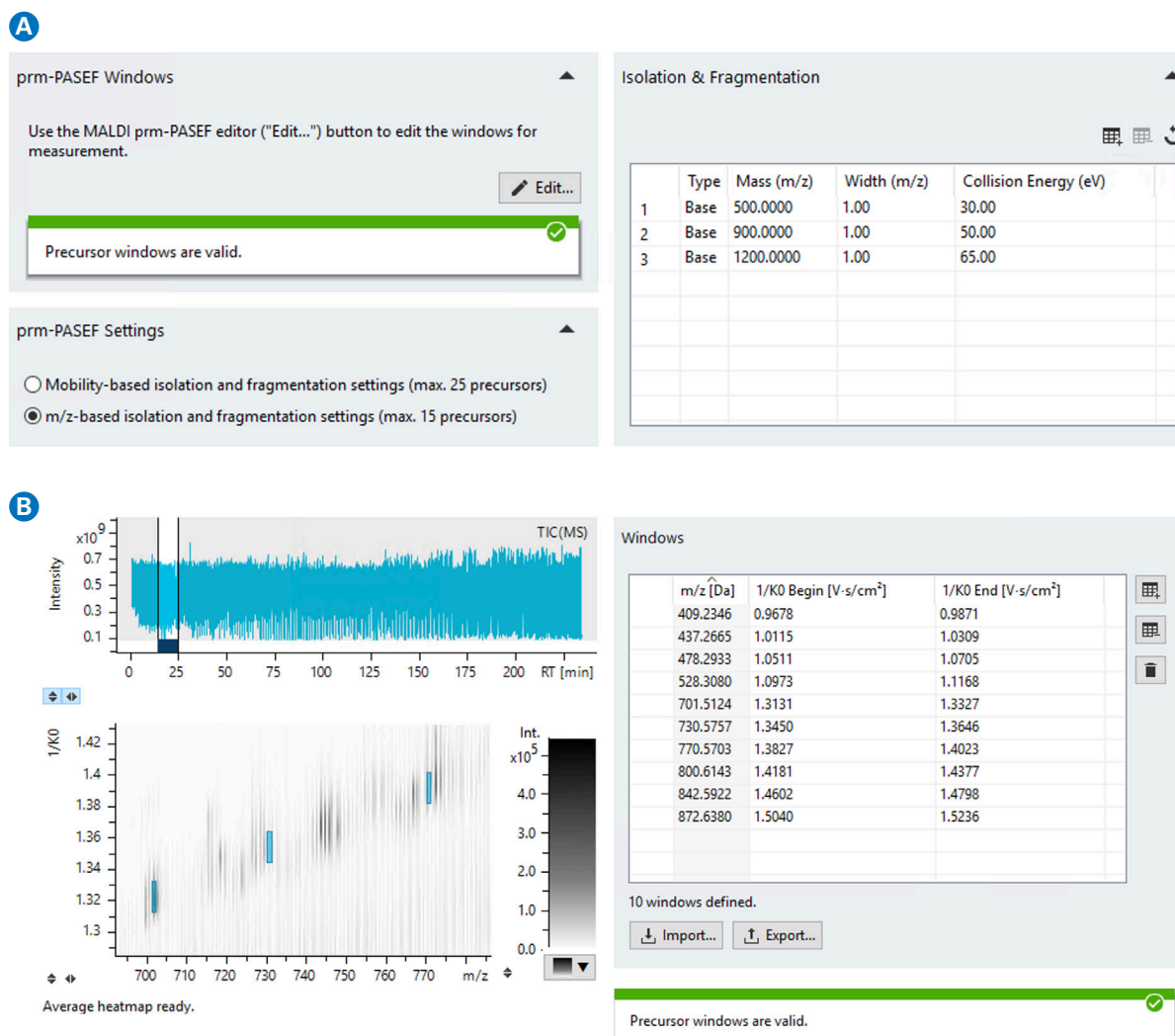


Figure 4. **A**) timsControl MS/MS page for the prM-PASEF Scan Mode to select and define the isolation and fragmentation modulation. In this example m/z -based settings were chosen. **B**) prM-PASEF window editor for the definition of the mobility isolation windows. Previous MS analysis can be loaded to display the overlay of the isolation windows as shown on the left, including a zoom-in view.

iprm-PASEF data analysis

After acquisition, the iprm-PASEF data was imported into SCiLS Lab, resulting in a new iprm-PASEF project. The MS/MS information of the 10 precursors is found in the feature navigator as resolved bands on the precursors $1/K_0$ windows. Again, T-ReX³ feature finding was applied to extract mobility-resolved features and images. Features that were found within both mass and mobility bounds of the isolation windows were specified as precursors, and features found outside the mass isolation width (but inside the mobility isolation window) were specified as fragments (See Figure 5A). The features of both types are hereby linked to the same isolation window and can be used to construct a mobility-resolved MS/MS spectrum.

Repeating the molecular annotation using the rule-based Lipid Species annotation tool in MetaboScape, the data now includes MS/MS information. For the feature at m/z 701.5117, previously annotated as PA 36:1, a molecular species annotation as PA 18:0_18:1 was reported based on m/z isotope pattern, CCS values, and MS/MS fragments matching.

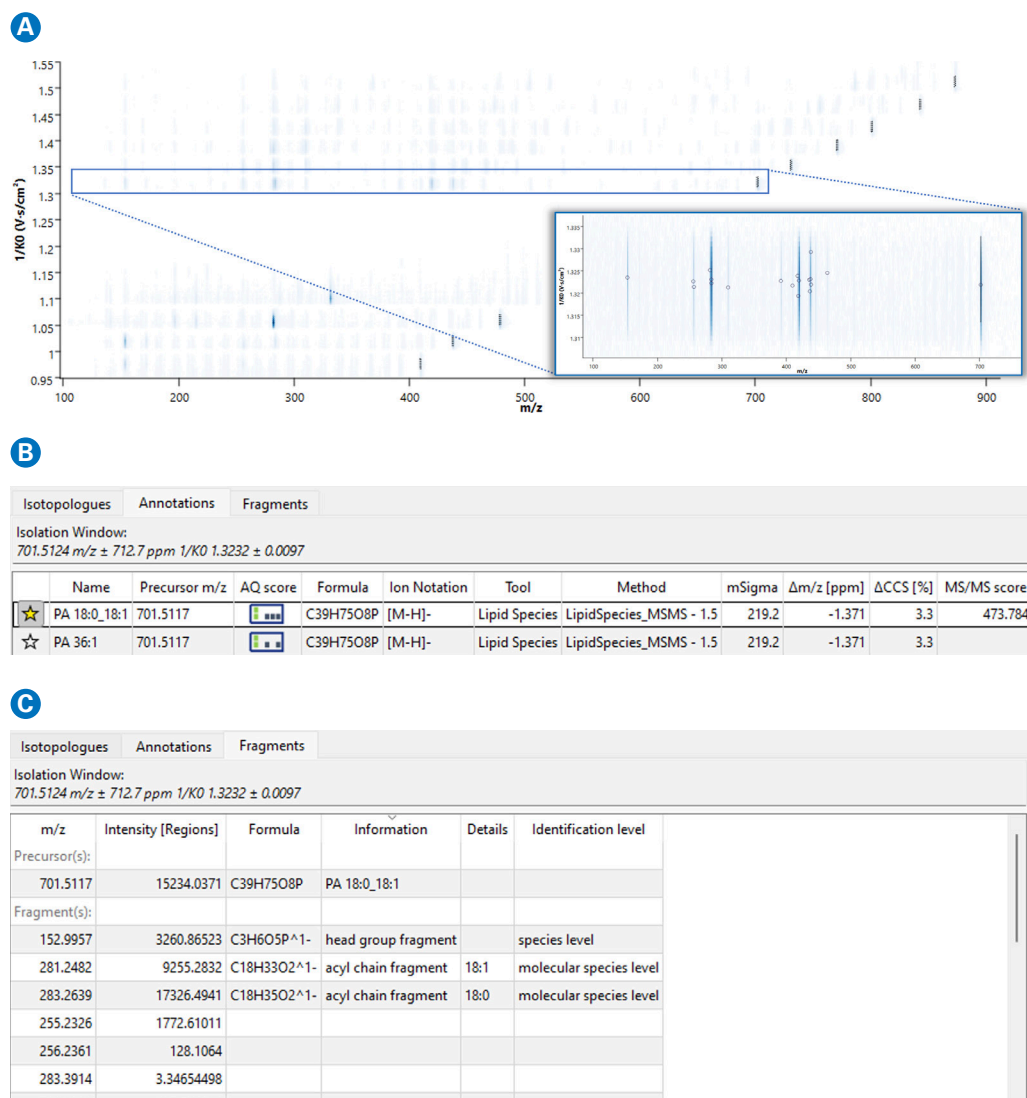


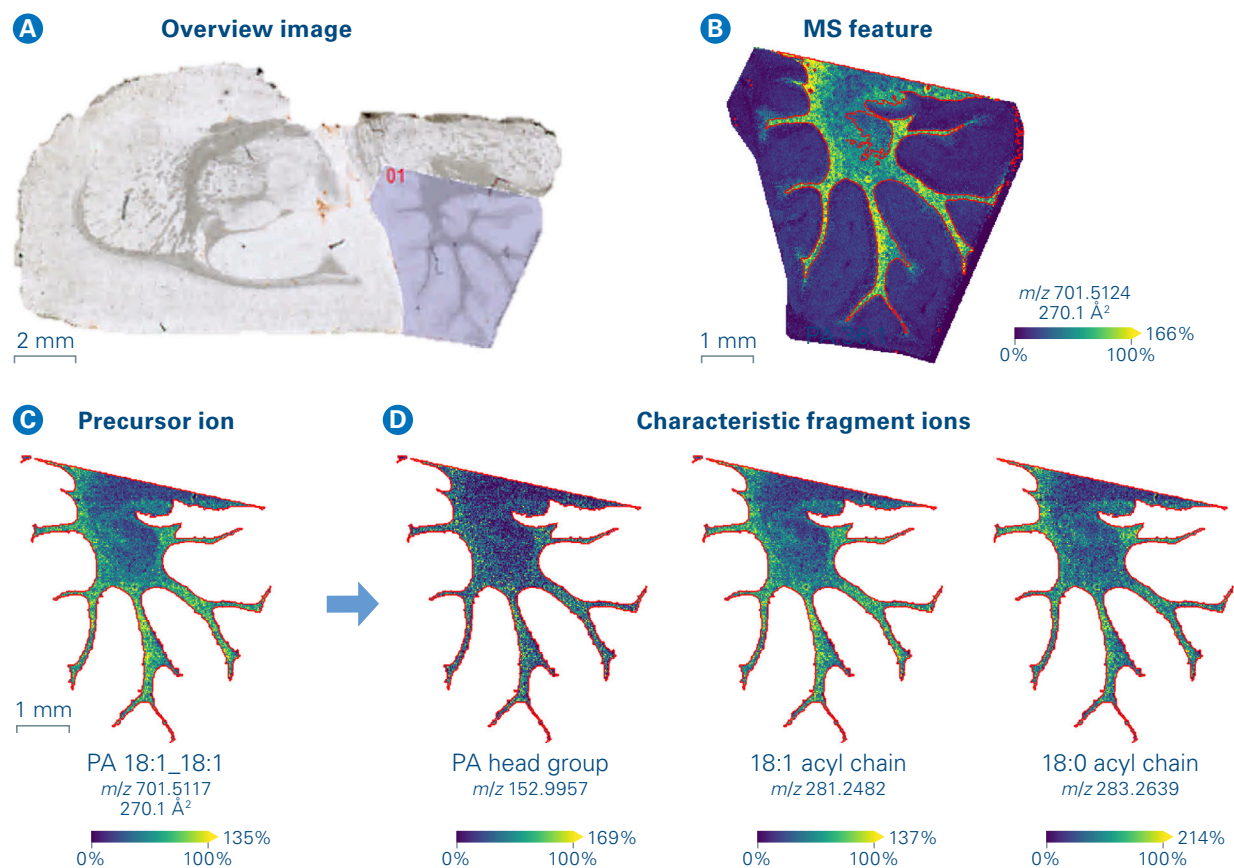
Figure 5. (A) iprm-PASEF feature navigator with zoom-in on the mobility isolation window for m/z 701.5124. (B) MS/MS annotations were summarized in the annotations explorer. (C) The fragments explorer lists the detected precursor and fragment features including fragment identity information on head group and acyl chains derived from the MetaboScape Lipid Species annotation.

Table 2. Annotated lipids from iprm-PASEF run, including m/z , CCS, and MS/MS scoring results.

exp. m/z	$1/K_0$ [V·s/cm ²]	CCS [Å ²]	Name	$\Delta m/z$ [ppm]	Δ CCS [%]	MS/MS score
437.2659	1.022	211.0	LPA 18:0	-3.4		830
478.2933	1.059	218.3	LPE 18:1	-1.3	0.8	966
528.3085	1.104	226.9	LPE 22:4	-2.1	1.0	945
701.5117	1.322	269.9	PA 18:0_18:1	-1.4	3.3	469
770.5689	1.389	283.2	PE 18:1_20:1	-2.1	3.5	640
730.5777	1.354	276.3	PE O-36:1	2.9	3.1	121
872.6335	1.522	309.6	PS 18:1_24:0	-5.8		643

This annotation was substantiated by the detection of the PA head group fragment and the 18:1 and 18:0 fatty acyl chain fragments (Figure 5). A molecular annotation on this level would not be valid based on m/z and CCS information alone. Enabled by the iprm-PASEF workflow, multiple MS/MS spectra were recorded within one imaging run, leading to a successful molecular annotation of 7 out of 10 precursors as listed in Table 2.

Compared to spot-based MALDI MS/MS profiling approaches, iprm-PASEF preserves the spatial information of the targeted compounds. Hence, a visualization of the precursor and fragment ion distribution provides additional information resulting in molecular annotation with greater confidence (Figure 6). For this example, the MALDI MS ion image was obtained for the whole rat cerebellum, while iprm-PASEF ion images exclusively depict the white matter which was exported as ROI for the respective analysis.

**Figure 6.** Ion images for PA 36:1 including (A) the sample overview image with measurement region, (B) the MS ion image and the iprm-PASEF images of (C) the precursor ion, and (D) the characteristic fragment ions of the PA head group and respective fatty acyl chains.

Conclusions

iprm-PASEF provides a start-to-end multiplexed MALDI MS/MS Imaging workflow, including the targeted acquisition and data analysis for a maximum of 25 precursors per imaging run. Advanced acquisition setups using subpixel definition allow for modulation or addition of the precursor selections or optimization of collision energy and isolation parameters for further investigation.

- CCS-enabled MALDI MS/MS Imaging for up to 25 precursors per run
- Accurate molecular identification while preserving the spatial information
- Full workflow solution using SCiLS Lab and MetaboScape-powered molecular annotation



For companion information on how to utilize the iprm-PASEF workflow, view this [webinar on demand](#).



Benefit

Feature

Advanced Data Analysis	Processing and analysis with next generation machine learning algorithms and API functionality
Integrated Imaging workflow	SCiLS Lab serves as the connection for automated run startup with SCiLS Autopilot, molecular annotations with MetaboScape®, and autogenerated OME-TIFF files for viewing in SCiLS Scope
Shareable Image Viewing	SCiLS™ Scope is a recent addition that enables collaborative viewing of a target list for non-expert users



Benefit

Feature

Identify	Add confidence to your IDs using annotation quality (AQ) scoring with CCS
Flexible Annotation Tools	Supports spectral libraries like MetaboBASE®, HMDB, MetaboBASE Plant, as well as custom libraries, rule-based lipid species annotation, in-silico fragmentation and CCS-Predict Pro
High throughput	Use MetaboScape to process large sample cohorts rapidly with the client-server based software .

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