QuPath integration into SCiLS Lab brings histology to statistical data analysis in a fast and easy way

Histological evaluation is a key aspect in MALDI Imaging data analysis to interprete results in a contextual manner

Abstract

The combination of sophisticated statistical tools for MALDI mass spectrometry imaging (MALDI Imaging) data analysis and histopathology information is needed for meaningful interpretation of multiplexed data sets. The integration of QuPath and SCiLS Lab provides an easy software platform that uses histomorphologically defined regions as a basis for statistical data analysis.

Introduction

MALDI Imaging provides researchers and pathologist with a tool for multiplexed spatially resolved molecular analysis of tissue samples. There are various applications of MALDI Imaging including for the detection of new molecular markers for diseases, finding discriminating differences between study groups or in the analysis of the distribution of target molecules like drugs. Striking is the ability to analyze

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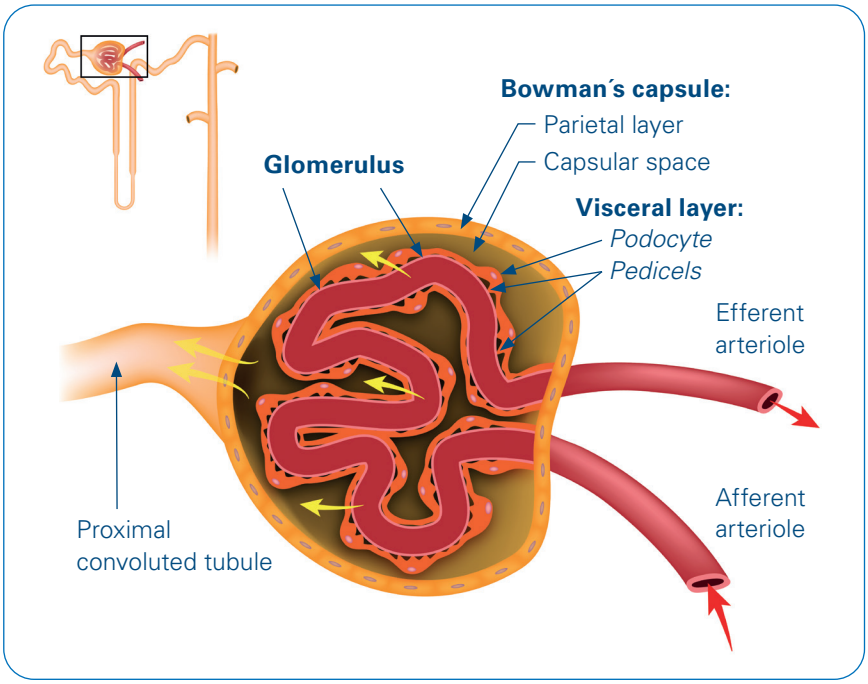


Figure 1: The renal corpuscule is the blood filtering unit of the nephron in the cortex of the kidney. It contains the Bowman's capsule, a sack-like structure with an outer parietal cell layer. The interior of the Bowman's capsule contains the glomerulus, a tuft of capillaries. Blood is filtered through the visceral layer which encloses the glomerulus. It is composed of podocytes that form filtering slits with their long foot processes called pedicles. The filtrate is collected in the capsular space and further processed along the nephron to form urin.

a multitude of compound classes such as metabolites, lipids, drugs, peptides, glycans and proteins. For the interpretation of this multidimensional data, it is important to link data analysis approaches to the underlying tissue histomorphology. QuPath is an open-source tool for pathololgy and bioimage analysis (1). Region annotations made in QuPath by histomorphological evaluation can be exported to SCiLS™ Lab with the QuPath to SCiLS Lab plug-in and can be used as the basis for statistical analysis for relevant interpretation of MALDI Imaging data.

Modern mass spectrometers, such as the timsTOF fleX, are capable of spatially resolving anatomical fine structures down to the singe cell level. Here, we demonstrate the possibilities of the QuPath integration to SCiLS Lab to find lipids associated with the renal corpuscules. These blood-filtering units of the nephron are on average 200 μm in diameter and contain a glomerulus, a cluster of capillaries embedded in a sack like structure, called the Bowman's capsule. The outer epithelium of the Bowman's capsule is formed by a parietal cell layer. The visceral layer encloses the glomerulus and is composed of podocytes and their pedicles which form with adjacent pedicals filtration slits. Mesanglial cells are located in the interior of the Bowman's capsule between the capillaries of the glomerulus (Figure 1).

Methods

Sample preparation

A frozen rat kidney sample was sectioned in a cryostat at 10 μm thickness and mounted on an IntelliSlide (Bruker Daltonics GmbH & Co. KG, Bremen, Germany). The slide was dried under vacuum and a grayscale reference scan was generated on a TissueScout Scanner (Bruker). 2,5-Dihydroxybenzoic acid (DHB) was applied by sublimation on top of the slide. Red phosphorous was spotted next to the section for external calibration.

Data acquisition

MALDI Imaging data was acquired on a timsTOF fleX instrument (Bruker) in positive Q-TOF mode in the mass range 300-1000 Da at 10 μm pixel size. A rectangular measurement area was defined in the renal cortex covering 27,350 pixels.

Single MALDI TIMS MS/MS spectra were collected from tissue for selected lipids to increase the annotation confidence. Final spectra were averaged from several MALDI events using 40 or 45 eV as collision energies.

Histological staining

After MALDI Imaging, the same section was stained with hematoxylin and eosin (H&E). First, matrix was removed by washing for 5 minutes in 70% ethanol. The slide was then stained using a standard protocol. A high resolution optical image of the section was generated on a Nanozoomer-SQ digital slide scanner

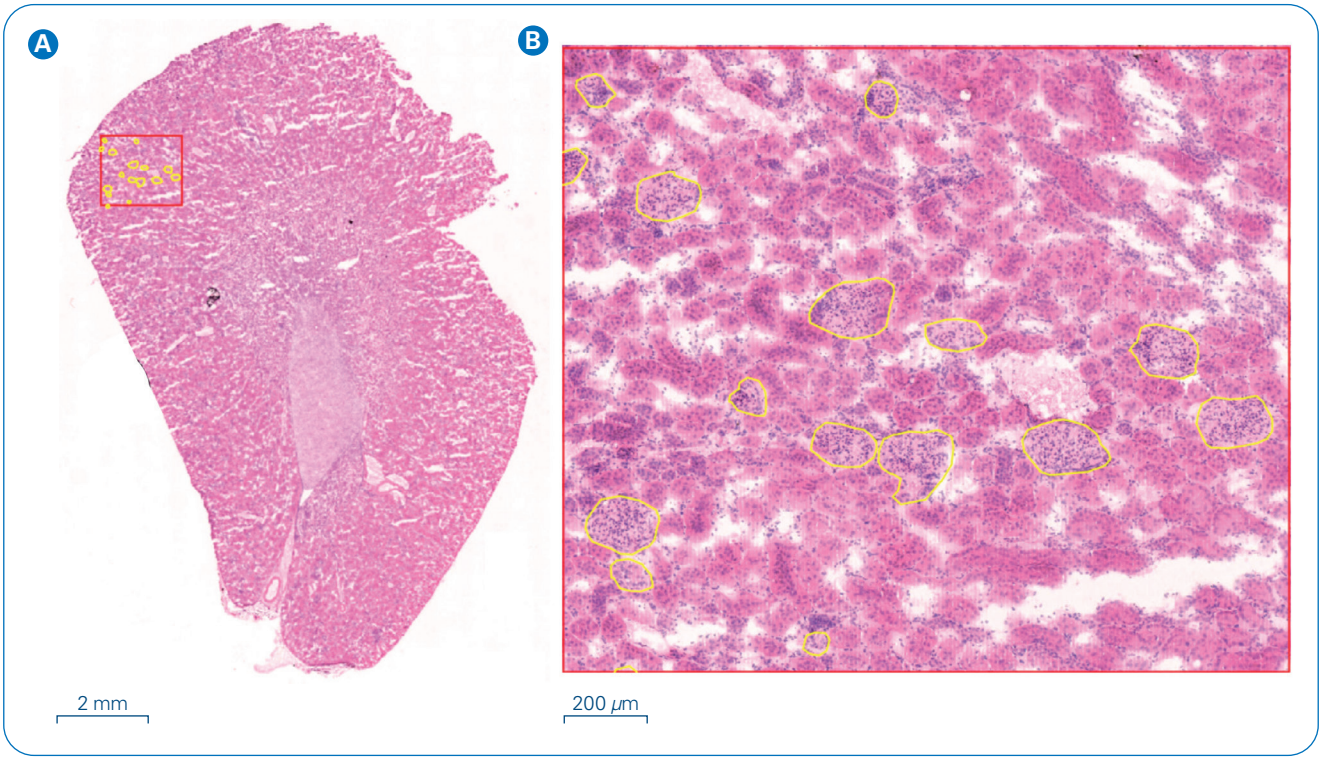


Figure 2: Region annotations in QuPath. Glomeruli were localized and borders defined manually with the QuPath polygon tool. Entire section (A) and zoom in of the MALDI Imaging measurement area (B). Glomeruli regions are delineated in yellow and the measurement area in a red rectangular.

(Hamamatsu Photonics, Hamamatsu, Japan). The 20x objective was selected to produce an image resolution of 0.46 µm/pixel. The output file *.ndpi can readily be opened with QuPath. However, other file formats are also supported (Supported image formats – QuPath 0.3.0 documentation).

Histological region annotation

The optical image file of the H&E-stained rat kidney section was visualized and annotated for histological regions with QuPath 0.3.2 [1]. QuPath offers different annotation tools to draw regions of interest including rectangular, ellipse, polygon, brush and wand tools. In this study, the polygon tool was used to define regions in the measurement area where glomeruli have been histologically localized. The glomeruli regions were then exported to SCiLS Lab 2022b (Bruker) via the "Export annotations to SCiLS Lab" extension which creates a *.sef file. The *.sef file can be imported to an existing SCiLS Lab file containing the MALDI Imaging data for this particular histological section.

MALDI Imaging data analysis

Imaging data were imported to SCiLS Lab 2022b and root mean square (RMS) normalized. The histological regions defined with QuPath were imported to SCiLS Lab. This step imports both the coordinates of the glomeruli regions and the H&E image. The H&E image was co-registered to the low-resolution reference scan of the section which is initially present in the SCiLS Lab file after Bruker data import. The image registration was performed with the SCiLS Lab co-registration tool.

Additionally, a segmentation analysis was performed on the RMS normalized data with the 151 most intense peaks with the bisecting k-means clustering algorithm and the correlation distance as the distance metric. Finally, the co-localized to region tool of SCiLS Lab was used to find lipids that accumulated in the glomeruli regions. In this case, the tool was applied to MALDI images derived from a list of automatically annotated lipids by MetaboScape®. However, the co-localized to region tool can principally be applied to any peak list in SCiLS Lab.

Lipid annotation with MetaboScape

To get a first hint on the identity of the lipids present in the kidney dataset, we performed an optional lipid annotation in the software MetaboScape. Therefore, MALDI Imaging data were imported to MetaboScape 2022.

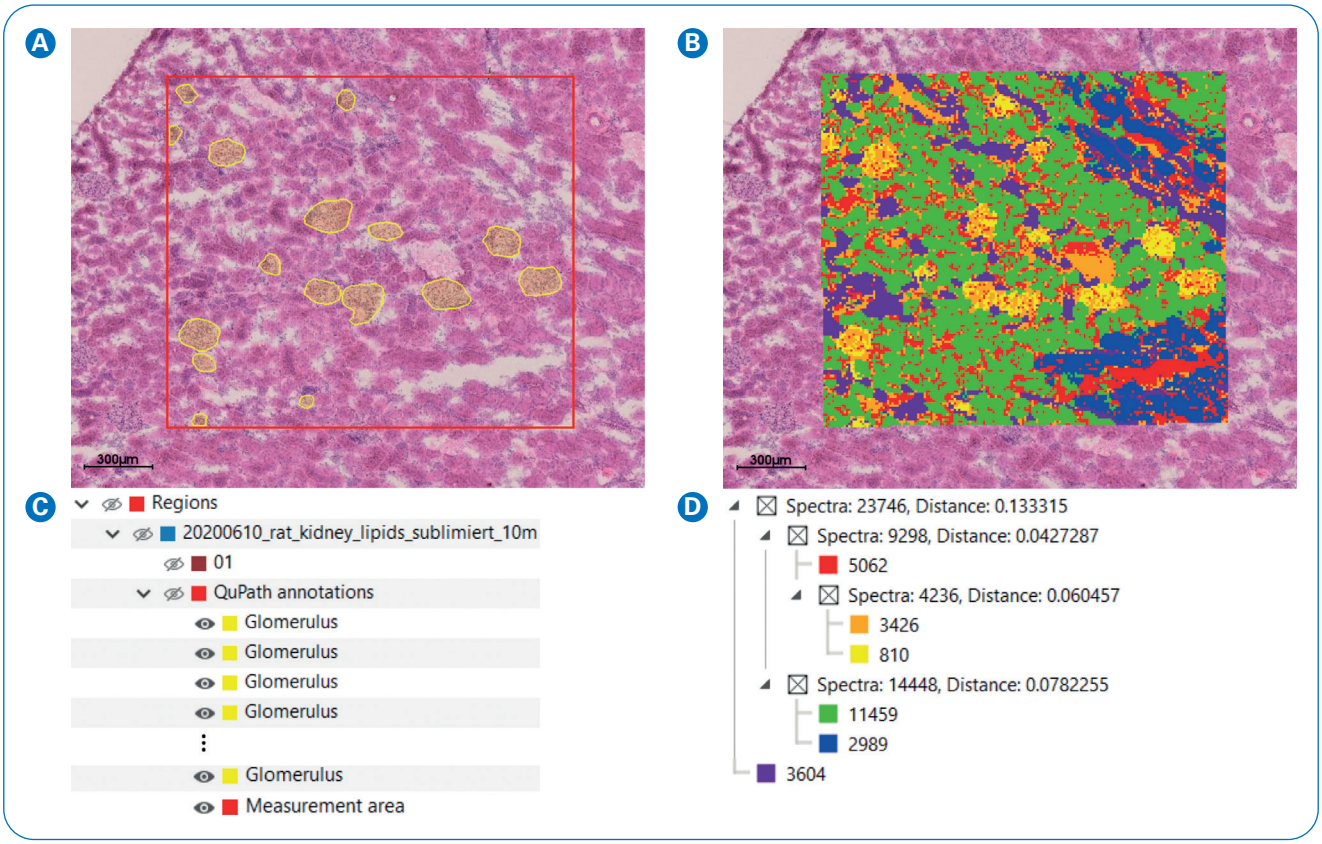


Figure 3: Glomeruli regions were imported from QuPath to SCiLS Lab and visualized (yellow regions in Ⓐ). The measurement area is indicated by a red rectangle. Imported regions are visible in the Visualization pane of the software in the Regions tree Ⓒ and can be used for all tools in the software. SCiLS Lab segmentation map Ⓑ and segmentation cluster tree Ⓓ assigning the same colors to pixels with similar spectra. Both graphics (Ⓐ and Ⓑ) are overlays with the corresponding H&E image of the same section used for MALDI Imaging analysis.

Spectral data were collected from four different regions derived from the segmentation map. As a maximum value, 200 spectra per region were imported. The T-ReX^2 algorithm was used for feature detection using $[M+H]^+$ ions as primary ion and $[M+Na]^+$ and $[M+K]^+$ as seed ions. The "Lipid Blast for MetaboScape" target list was used to annotate lipids according to exact mass with a mass error tolerance of 5 ppm. In addition, the Lipid Species tool was applied to account for PCs, PEs and SMs as lipid classes again with a mass error tolerance of 5 ppm.

For interpretation of on-tissue MALDI TIMS MS/MS data, DataAnalysis version 5.3 was used to create *.mgf files after deisotoping. These *.mgf files were added to the respective ion features in MetaboScape to allow exploitation of the tools in MetaboScape to analyze the fragment spectra.

Results

Linking histology with MALDI Imaging data in SCiLS Lab

The QuPath to SCiLS plug-in was used to integrate histological region annotations with statistical analysis of MALDI Imaging data. As a first step, glomeruli regions were defined in QuPath. Figure 2 shows a graphic of the H&E image of the rat kidney section after MALDI Imaging data acquisition and subsequent staining as well as a zoom on the measurement region. The glomeruli regions are delineated in yellow, and the measurement area is provided in red.

The QuPath to SCiLS extension provides the ability to export annotated regions to SCiLS Lab for statistical data analysis. Here, we exported the glomeruli regions to SCiLS to link the histological annotations with the MALDI Imaging data. This is important as correct interpretation of MALDI Imaging data relies on the histology of the tissue. SCiLS tools such as discriminant analyses or the colocalization tool can now easily be performed on regions derived from histopathological evaluation. Moreover, unsupervised segmentation maps can be compared to the histology in the same software.

Figure 3A displays the exported glomeruli regions in SCiLS Lab. The

glomeruli regions occur also in the SCiLS Region Tree after export from QuPath (3C). SCiLS Lab automatically generates mean spectra for all newly imported regions as well as the "root" region (named "QuPath annotations" in Figure 3C). Mean spectra can easily be visualized and compared to other regions by the user if desired.

An unsupervised segmentation analysis also grouped glomeruli pixels in one cluster (Figure 3B, pixel marked in yellow). This analysis is based on spectral similarity within the MALDI Imaging data and does not require any *a priori* information provided by the user. The algorithm assigns the same color to similar spectra, i.e. pixel. The outcome can be compared to the histological annotation and be considered as an independent validation for the manual region annotation. In addition, this shows that glomeruli regions are characterized by a distinct spectral pattern or, in other words, express a number of glomeruli specific lipids. The segmentation analysis results, in addition to the segmentation map, in an interactive cluster tree (3D).

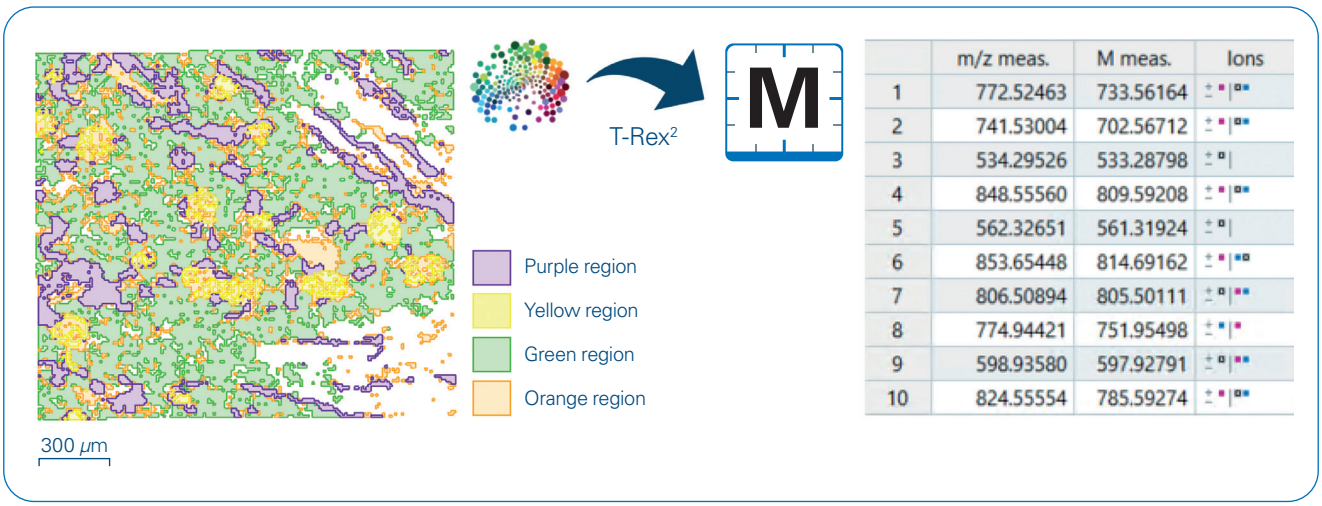
Automatic lipid annotations with MetaboScape

The goal of this study was to determine which lipids occur specifically in glomeruli regions through the use of annotations in MetaboScape. In this context, the term "lipid annotation" refers to the assignment of names to lipids based on exact mass matching. Lipid annotation is therefore an initial strategy to retrieve meaningful information on *m/z*-features in MALDI Imaging.

Spectra from four different regions derived from the segmentation approach in SCiLS Lab were loaded into MetaboScape (Figure 4). The T-ReX^2 recursive feature extraction algorithm was applied on the data resulting in a table consisting of 202 features. Several adducts were merged into one feature in MetaboScape (Figure 4). From the total of 202 features, we could annotate 49 features tentatively comprising phosphatidylcholines (PCs), phosphatidylethanolamines (PEs) including lysoPEs and sphingomyelines (SMs).

Lipids occurring in renal glomeruli

The lipid annotations were then exported back to SCiLS Lab. To determine which lipids occur specifically in the glomeruli, the colocalization to region tool in SCiLS Lab was applied and distributions visualized for lipids with a correlation factor higher than 0.25 (Table 1, Figure 5). The colocalization tool uses an image mask, in this case the glomeruli region annotations (see Figure 5, panel 0) and compares this mask against the ion distribution maps. The distribution maps of eight ion species including adducts, or six different lipids correlated well with the glomeruli regions (Figure 5). The highest correlation showed a putative PC 36:2, followed by protonated and potassiated SM 40:1;O2 which localized with high abundance in glomeruli. The annotation of SM 40:1;O2 was confirmed by on-tissue MALDI TIMS MS/MS (data not shown).



	m/z meas.	M meas.	Ions
1	772.52463	733.56164	
2	741.53004	702.56712	
3	534.29526	533.28798	
4	848.55560	809.59208	
5	562.32651	561.31924	
6	853.65448	814.69162	
7	806.50894	805.50111	
8	774.94421	751.95498	
9	598.93580	597.92791	
10	824.55554	785.59274	

Figure 4: Import of the MALDI Imaging spectral data from SCiLS Lab to MetaboScape 2022a. A maximum of 200 spectra from four regions derived from the segmentation map were selected for the import. MetaboScape produces a feature table by the T-ReX^2 recursive feature extraction. This algorithm performs de-isotoping and merges different adducts from the same ion species into one feature. Features can then be annotated by different tools in MetaboScape.

Number	*m/z*	Name	NeutralMass	Notation	Formula	Δ*m/z* [mDa]	Correlation value
1	518,3212	lysoPE 19:0	495,3321	[M+Na]+	C24H50NO7P	-0,55	0,28
2	546,3527	lysoPE 21:0	523,3636	[M+Na]+	C26H54NO7P	-0,1	0,26
3	796,5246	PC 34:2	757,5611	[M+K]+	C42H80NO8P	-0,02	0,29
4	824,5555	PC 36:2	785,5927	[M+K]+	C44H84NO8P	-0,1	0,45
5	731,606	SM 36:1;O2	730,5985	[M+H]+	C41H83N2O6P	-2,07	0,29
6	753,5875	SM 36:1;O2	730,5985	[M+Na]+	C41H83N2O6P	-0,67	0,3
7	809,6499	SM 40:1;O2	786,6607	[M+Na]+	C45H91N2O6P	-0,67	0,27
8	825,6237	SM 40:1;O2	786,6606	[M+K]+	C45H91N2O6P	-1,75	0,34

Table 1: Tentatively assigned lipids found in the glomeruli regions. The given correlation value is a measure how well the ion image correlates with the region annotation (correlation value = 1, perfect correlation; correlation value = 0, no correlation; correlation value = -1, perfect anti-correlation).

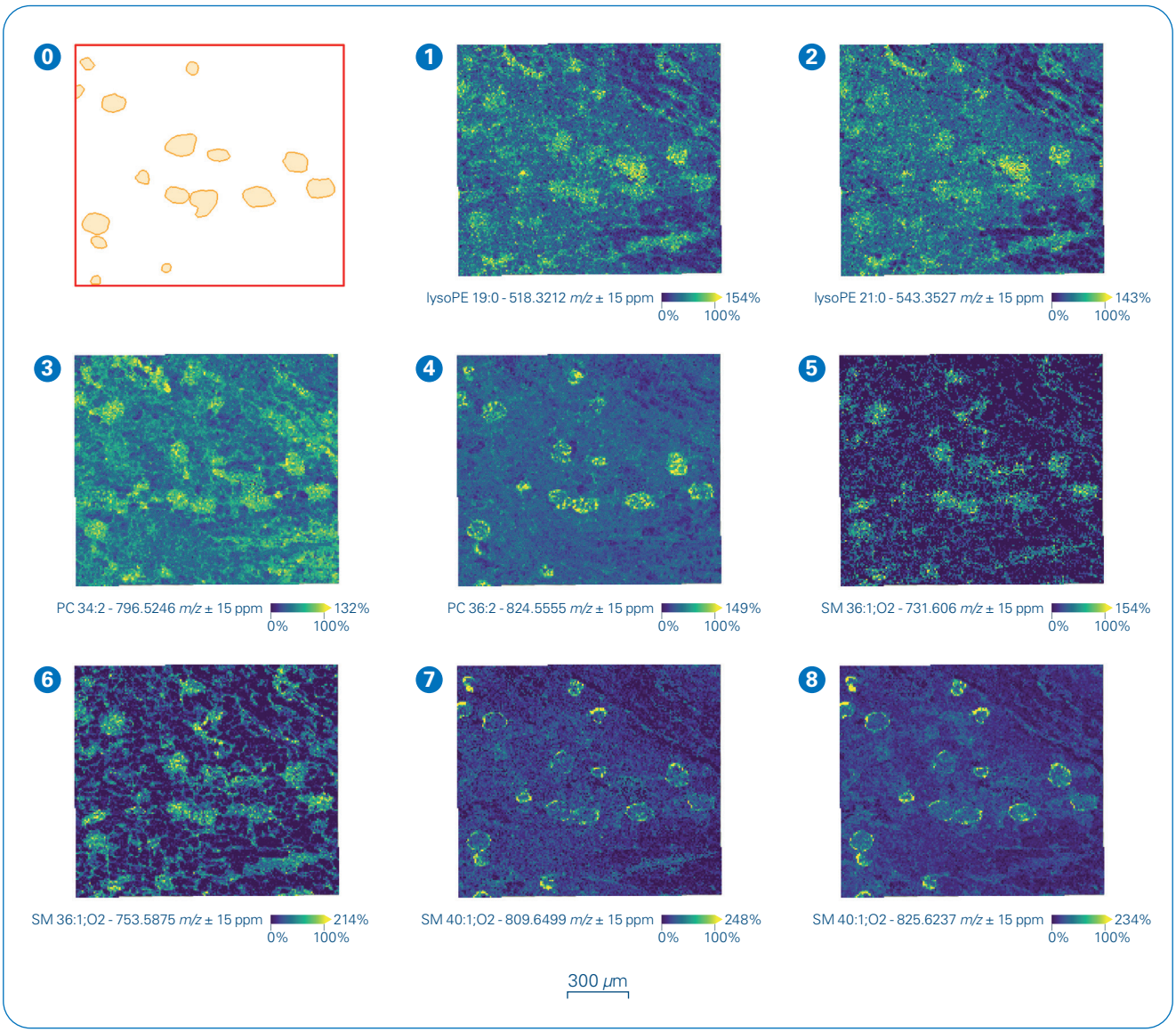


Figure 5: Lipid ion species colocalizing with annotated glomeruli regions as defined by QuPath (panel 0). The numbering corresponds to the order listed in Table 1.

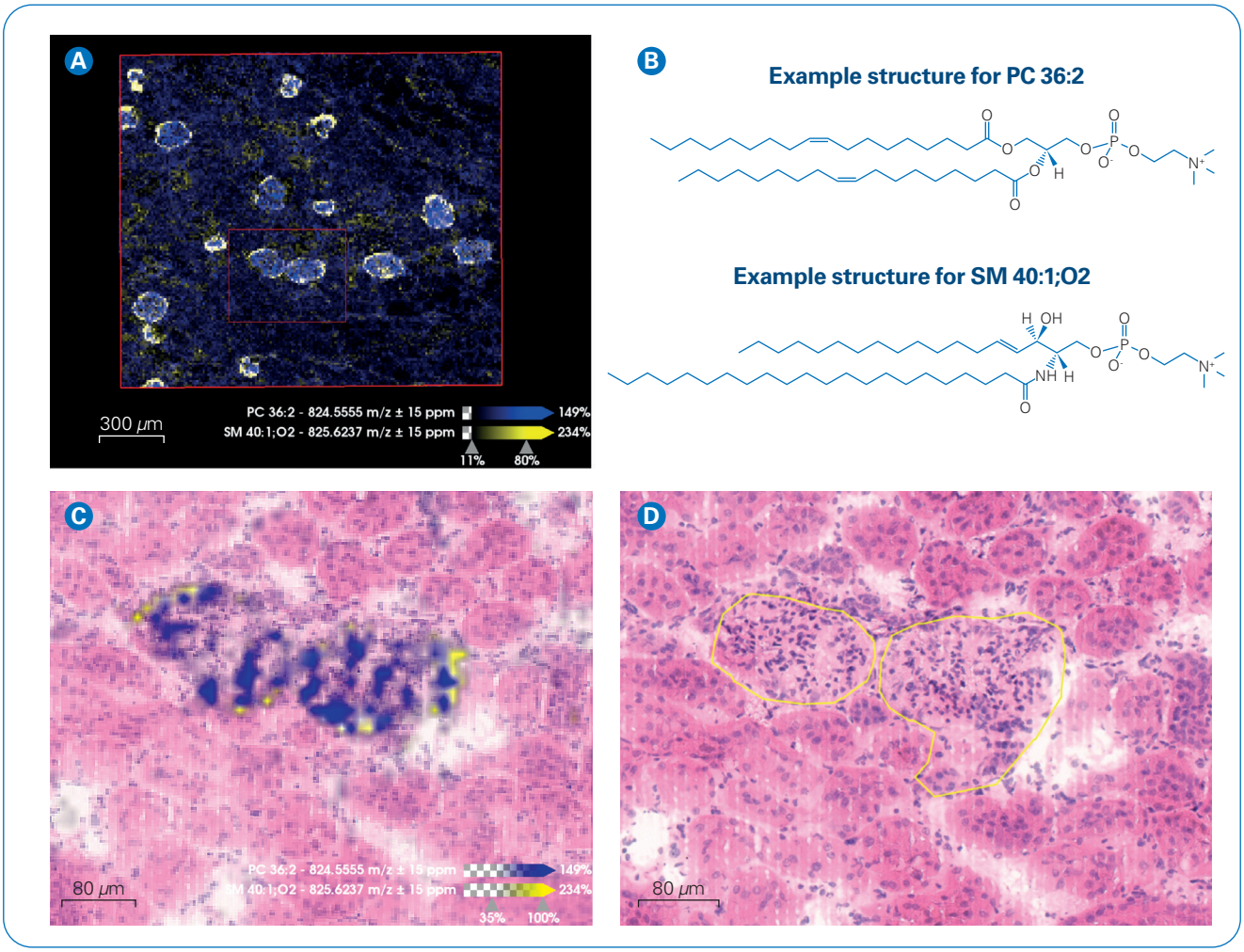


Figure 6: Lipids in the renal corpuscles. Two-channel image of a putative PC 36:2 (in blue) and SM 40:1;O2 (in yellow) in the cortex of the measured rat kidney sample (A). Enlarged view of two renal corpuscules as indicated in the inlet in A (C). Low intensity pixels were made transparent. H&E stain and region annotations in QuPath for the same area (D). SM 40:1;O2 (yellow) was present in the parietal layer of the Bowman's capsule while PC 36:2 was mainly found in the interior of the renal corpuscle. Example structures for both compounds, PC 18:1/18:1 and SM 16:1/22:0 are shown in (B).

In a two-channel image merging both ion distributions, it becomes obvious that SM 40:1;O2 was mainly localized in the parietal layer of the Bowman's capsule (Figure 6). The putative PC 36:2, however, was found in high abundance in the interior of the Bowman's capsule, i.e., Mesangium, Glomerulus capillaries, and visceral layer of the Bowman's capsule (Podocytes). The high spatial resolution capabilities of the timsTOF fleX made it possible to distinguish fine anatomical structures like the outer and inner part of the renal corpuscule. In summary, the QuPath integration to SCiLS allows interpretation of lipid distributions measured by MALDI Imaging in a histological context.

Conclusion

- The QuPath to SCiLS plug-in provides an integrated software solution to connect MALDI Imaging data with histology.
- The SCILS Lab tools for statistical data analysis makes it easy to find compound distributions associated with relevant histopathological regions.

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Reference

[1] Bankhead P et al. (2017). *QuPath: Open source software for digital pathology image analysis.* Scientific Reports. https://doi.org/10.1038/s41598-017-17204-5

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