

Correlating metabolites, lipids, and N-glycans in murine kidney microstructures via integrative mass spectrometry imaging

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Understanding how metabolites, lipids and glycans interact within tissues at the same cellular layer is key to uncovering their roles in health and disease. Herein, we present an integrative multi-omic mass spectrometry imaging (MSI) approach correlating metabolites, lipids and N-glycans, including their linkage-specific sialic acid information, in a single renal tissue section. Our protocol enables the sequential detection of hundreds of metabolites and lipids species alongside diverse glycosylation traits in small cell clusters across the kidney. Importantly, our pixel-to-pixel co-registration and clustering correlates glycans (recorded in positive mode MS), metabolites, and lipids (both in negative mode MS) across kidney microstructures. This analysis reveals strong association between taurine, TCA metabolites, sulfatides, and polyunsaturated phospholipids, and specific sialic acid linkages on N-glycans in the thick ascending limb and collecting ducts. The robust strategy maximizes molecular insights from scarcely available samples and offers a powerful tool for exploring tissue complexity in both normal and disease states.

The spatial organization of cells in a tissue determines their behavior, differentiation, and roles in both homeostasis and pathological processes. The molecular profile of a cell is shaped by its interactions with neighboring cells, making spatial molecular characterization vital for understanding disease mechanisms. Mass spectrometry imaging (MSI) has emerged as one of the cornerstones of spatial biology due to its ability to perform untargeted analysis of multiple molecular classes, such as metabolites, lipids, peptides, proteins, and glycans from tissue sections. Diseases like cancer involve complex biochemical changes across all these molecular layers, which are often interconnected. For example, cancer cells take up significantly more glucose for glycolysis as compared to normal cells, and the common oncogenic signaling pathway is characterized by an exacerbated lipogenesis, where fatty acids are synthesized from carbohydrates and stored as triglycerides

(TGs)¹. Amino acids and sugar nucleotide levels affect protein and lipid glycosylation^{2,3}, which in turn impacts, amongst others, lipoprotein metabolism⁴ and cancer progression^{5–7}.

While MSI, in theory, can provide spatial information across many molecular classes, practical limitations such as tissue preparation, technical constraints, and focus of the lab often restrict analysis to a single omics layer. To achieve true multi-omics integration, i.e., mapping biomolecular identities and abundances to specific cell populations, the sequential analysis of multiple molecular classes from the same tissue section is required, followed by an integrative data analysis to connect the molecular classes in a biologically meaningful manner. Spatial resolution of the imaging determines the extent to which individual cells can be resolved as distinct entities, with cell dimensions typically between 50 (e.g., length of endothelial cell) and 5 (e.g.,

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diameter of a lymphocyte) μm . Prior studies have attempted sequential multi-omic analysis using either formalin-fixed paraffin-embedded (FFPE) tissues^{8,9} or fresh-frozen tissues, but not without limitations. While lipid analysis can be performed from FFPE tissues, they undergo cross-linking and a series of modifications during fixation, making it an unreliable source for quantitative analysis^{10,11}. The use of fresh-frozen material is therefore paramount in performing sequential metabolite and lipid imaging. Three- and even eight-layer multi-omic analysis has recently been shown from a single fresh-frozen tissue section, resulting in information-rich molecular characterization of murine brain and rabbit liver samples. However, these approaches suffered from limitations in the number of analytes detected, especially for the labile sialylated N-glycans, as well as high tissue damage throughout the acquisitions and a relatively low spatial resolution ($>30 \times 30 \mu\text{m}$ pixels)^{12–14}, limiting the capabilities of discriminating between small cell clusters that define microanatomical structures. Additionally, the full potential of the complex multi-layer data was not yet used to study spatial biology from a multi-omic perspective.

Here, we developed a multi-omic sample preparation protocol enabling spatial mapping of metabolites, lipids (negative mode MSI), and N-glycans (positive mode MSI after sialic acid derivatization) from the same fresh-frozen tissue section with a $20 \times 20 \mu\text{m}$ spatial resolution, allowing the integrated molecular characterization of small cell clusters. Our protocol, as compared to other multi-omic MSI initiatives, demonstrates the comprehensive detection of metabolites and the detection of sialylated N-glycans, providing sialic acid linkage-specific information, critical in e.g., immune cell interactions, viral infections, and cancer¹⁵. We applied the optimized method to healthy

murine kidney sections, providing a relevant model system with highly complex and heterogeneous tissue morphology. Pixel-to-pixel co-registration of the omics layers and subsequent clustering analysis enabled studying the correlation between metabolites, lipids, and N-glycans in microstructures of the murine kidney. Next to the increased integrative potential of the data, our approach maximizes the amount of molecular information extracted from limited available tissue specimens.

Results

We developed a multi-omic MSI experimental and data analysis approach integrating metabolites, lipids, and N-glycan images from the same tissue section (Fig. 1). Applying the method on fresh-frozen murine kidney tissues as a complex model system ultimately allowed to identify and relatively quantify 48 metabolite features, 161 lipid features, and 74 N-glycan features from the same tissue section with $20 \times 20 \mu\text{m}$ resolution. Crucial steps in the sample preparation included an ammonium formate washing step after metabolite analysis and on-tissue fixation prior to sialic acid derivatization for N-glycan analysis¹⁶ as detailed below. The workflow can be completed within 4 consecutive days, including instrument run time. Following lipid analysis and on-tissue fixation, the workflow can be paused for several days without compromising N-glycan analysis. Sensitivity of the MS measurements was maximized by using dedicated matrices and m/z ranges for metabolites and lipids. Integrated data analysis was aided by precise pixel-to-pixel co-registration of the different omics layers based on morphological features of the tissue.

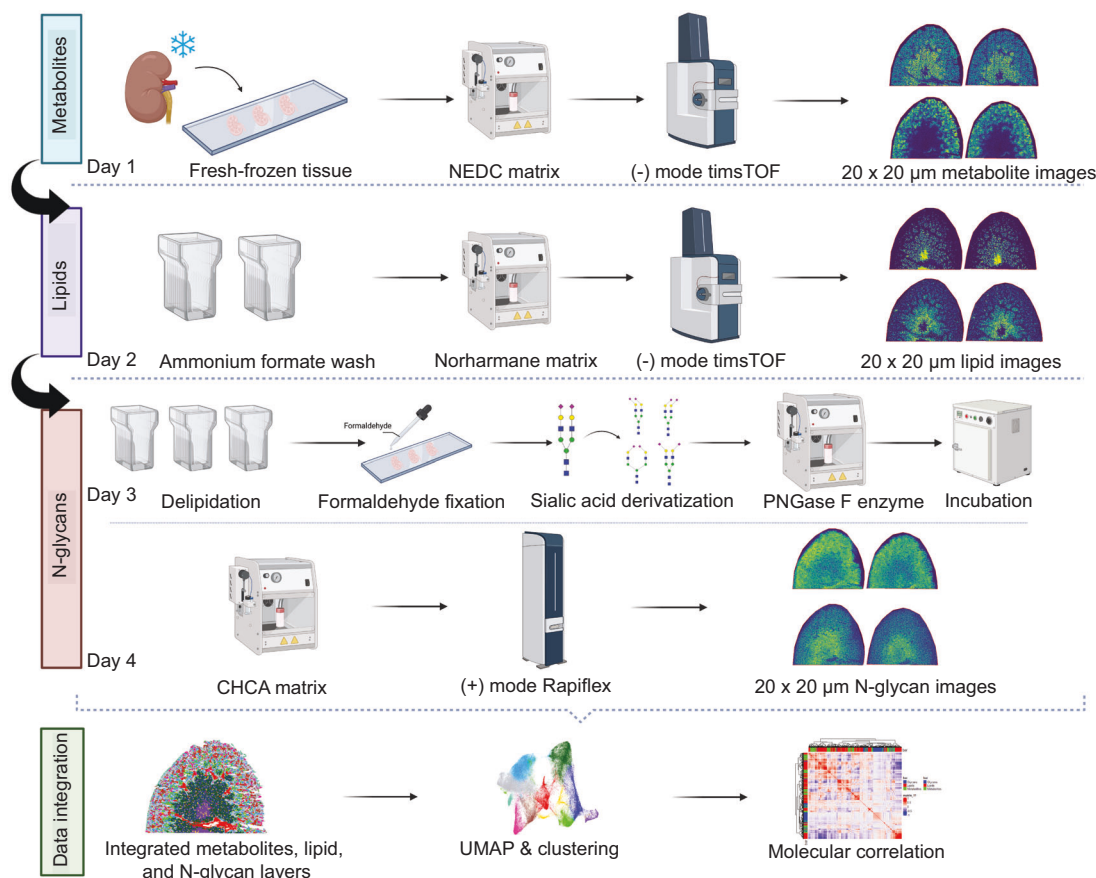


Fig. 1 | Multi-omic MALDI-MSI workflow. Optimized protocol for sequential analysis of metabolites (negative ion mode), lipids (negative ion mode), and N-glycans (positive ion mode) from single fresh-frozen kidney sections mounted on

an ITO slide. The entire process, including the analysis of all molecular classes, takes up to 4 days. Created in BioRender. Wührer, M. (2026) <https://BioRender.com/dxbsbsbl>.

Maximizing lipid and metabolite coverage from a single tissue section

By evaluating the matrix and tuning the timsTOF flex-MS ion transfer configuration, we ensured the broadest metabolic and lipidomic coverage from a single tissue section (Fig. 2). *N*-Naphthylethylenediamine dihydrochloride (NEDC) and norharmane (NOR) are matrices particularly known for the spatial characterization of metabolites and lipids by MALDI-MSI in negative ion mode. For metabolite imaging, NEDC outperformed NOR (m/z 50–1350) both in terms of the total number of metabolite features detected and their absolute intensities (Fig. 2a). The coverage of the lower mass metabolites was further improved by tuning the instrumental parameters for NEDC in the recorded m/z range (50–650), resulting in a total of 48 metabolite features detected with putative IDs below 5 ppm mass error (Supplementary Data 1). Limiting the mass range and consequently lowering the radio frequency (RF) amplitude settings particularly improved the coverage and intensities of carboxylic acid and amino acid ions. The metabolites were searched against KEGG pathway analysis (Fig. 2b), with the majority of the features corresponding to the tricarboxylic acid cycle (TCA) and alanine, aspartate, and glutamate metabolism. For lipid analysis, using NOR as a matrix resulted in the detection of a more diverse repertoire of lipid subclasses as compared to using NEDC. When limiting the m/z range from m/z 50–1350 to 300–1350, the number of detected lipid features, including sulfatides (SHexCer), phosphatidylglycerols (PGs), phosphoinositides (PIs and PIPs), Gangliosides (GM) cardiolipins (CLs), increased from 142 to 161 (Fig. 2c and Supplementary Data 1) due to improved RF settings and the exclusion of a high intensity signal at m/z 167.062 suppressing other ions (Supplementary Fig. 1). The higher coverage of the lipids was independent of the prior metabolite analysis (Supplementary Fig. 2). Yet, up to a 10-fold increase in intensities was observed for the majority of lipid species, excluding PIs, when comparing the sequential analysis to the single lipid analysis under the same conditions (Fig. 2d and Supplementary Fig. 2). This was induced by the introduction of an ammonium formate wash. Of note, spatial resolution is maintained after the ammonium formate wash for the different lipid subclasses (Supplementary Fig. 3). For example, high abundance signals of m/z 331.262 [FA(22:4)-H][−], m/z 878.604 [SHexCer(40:1;O3)-H][−], and m/z 909.550 [PI(40:6)-H][−] colocalize with the glomeruli, the inner stripe of the outer medulla (ISOM) and outer stripe of the outer medulla (OSOM) and cortex, respectively, which is preserved in the sequential analysis (Fig. 2e).

Integrating N-glycans and sialic acid linkage information in the multi-omic analysis of mouse kidney

To enable the sequential analysis of N-glycans after metabolite and lipid imaging on the fresh-frozen material, we opted for RapifleX analysis due to superior sensitivity (Supplementary Fig. 4) and implemented on-tissue fixation with 10% formalin after delipidation. This resulted in a, on average 3-fold intensity increase of the N-glycan signals, largely independent of the N-glycan sub-classes or structural features (Fig. 3a and Supplementary Fig. 5). Using the optimized procedure, we identified 74 N-glycan features (Supplementary Data 2), covering oligomannose- and complex-type glycans, as well as (multi) fucosylated glycans and glycans carrying *N*-acetyl or *N*-glycolyl neuraminic acids (NeuAc; NeuGc). Importantly for the latter, we stabilized sialylated species and differentiated between sialic acid linkages by applying our previously established sialic acid derivatization protocol¹⁶. This enabled the analysis of sialylated N-glycans in a multi-omic MSI approach with sialic acid linkage resolution. The on-tissue fixation improved the spatial resolution of the N-glycan imaging (Fig. 3b), obtaining similar quality data to what was observed for FFPE material, which is the golden standard in N-glycan MSI^{9,16–22}. We found the largest differences in N-glycan distributions between the cortex and the outer medulla¹⁸. The medulla and, in particular, the ISOM were

characterized by high levels of oligomannose N-glycans (Fig. 4a–c), which were least processed (thus carrying a higher level of mannoseylation) in the OSOM (Fig. 4d). The cortex, on the other hand, was rich in highly fucosylated complex-type N-glycans (Fig. 4e). Furthermore, the complex-type glycans within the medulla displayed a higher level of LacNAc repeats as compared to the rest of the tissue (Supplementary Fig. 6). Importantly, our protocol allows the differentiation between α 2,3- and α 2,6-linked sialylated species and we indeed found that the α 2,3-linked sialic acids are abundant in ISOM, whereas α 2,6-linked sialic acids are the most abundant sialic acid linkage throughout the tissue and particularly pronounced in the cortex (Fig. 4f, g).

Multi-omic data integration and molecular correlation

Each molecular class (metabolites, lipids, and N-glycans) individually distinguishes the primary histological regions of the kidney, including the cortex, OSOM, ISOM, and large vessels (Fig. 4a, b, and Supplementary Figs. 7–10). To study the synergistic effects of multi-omic data analysis for the characterization of more refined tissue structures and to investigate correlations between molecular classes, we performed a pixel-to-pixel co-registration of the three data layers, based on anticorrelated ions. The quality of co-registration was further assessed by calculation of the normalized mutual information (NMI) (Fig. 4h and Supplementary Fig. 11). Any translation or rotation of the lipidomic or glycomics datasets relative to the metabolomic dataset resulted in lower NMI values, demonstrating the high accuracy of the achieved co-registration.

Analysis of the lipidomics data alone resulted in the most accurate clusters corresponding to tissue structures as annotated by a pathologist, including glomeruli, distal tubuli, proximal tubuli, blood vessels, collecting ducts, OSOM_thick ascending limb (TAL), and ISOM_TAL (Fig. 5a–i). Signature lipids of the individual clusters were used to corroborate the identity of the kidney tissue structures based on the well-validated data from the kidney lipid atlas (Fig. 4e)^{23,24}. The identity of the proximal and distal tubuli were further confirmed by multiplexed immunofluorescence directly following lipidomics MSI. The immunofluorescence was stained with LTL for proximal tubuli, against Calbindin for distal tubuli and with Hoechst for nuclei, and confirmed our distinct microstructural lipid signatures and cluster annotations (Fig. 5e–j).

The lipidomics clustering served as the tissue blueprint on which the glycomics and metabolomics layers were mapped (Fig. 5d). For example, blood vessels and blood rich regions were distinguished from glomeruli based on the combined information of all omics, in particular through high α 2,3-/ α 2,6-sialylation ratio and low fucosylation of complex-type N-glycans in addition to the presence of heme in the blood vessels (Fig. 5d and Supplementary Fig. 12). These observations highlight the relevance of the multi-omic characterization at high spatial resolution to define microanatomical tissue features composed of small cell clusters.

Additional principal component analysis (PCA) of the integrated data revealed further subgroups within e.g., the collecting ducts (CD). The identity of this cluster was supported by the highly specific and previously described marker lipid SHexCer 42:0;O4 m/z 924.646 (Fig. 4e)^{23,24}. The CDs were divided into two subgroups separated by PCI, showing a difference between CDs present in the ISOM as compared to the rest of the tissue (Fig. 6a–c and Supplementary Figs. 13–15). The ducts in the ISOM carry higher levels of sulfatides, the metabolites taurine, serine, and phosphoserine, as well as oligomannose and α 2,3-linked sialylated N-glycans. The other group of CDs displayed higher levels of PIP lipids, poly-unsaturated phospholipids, carboxylic acids, and fucosylated as well as α 2,6-linked sialylated N-glycans. This indicates that the sequential integration of metabolomics, lipidomics, and N-glycomics data yields synergistic insights into the tissue's fine structure, surpassing the level of detail obtained from each layer when analyzed independently.

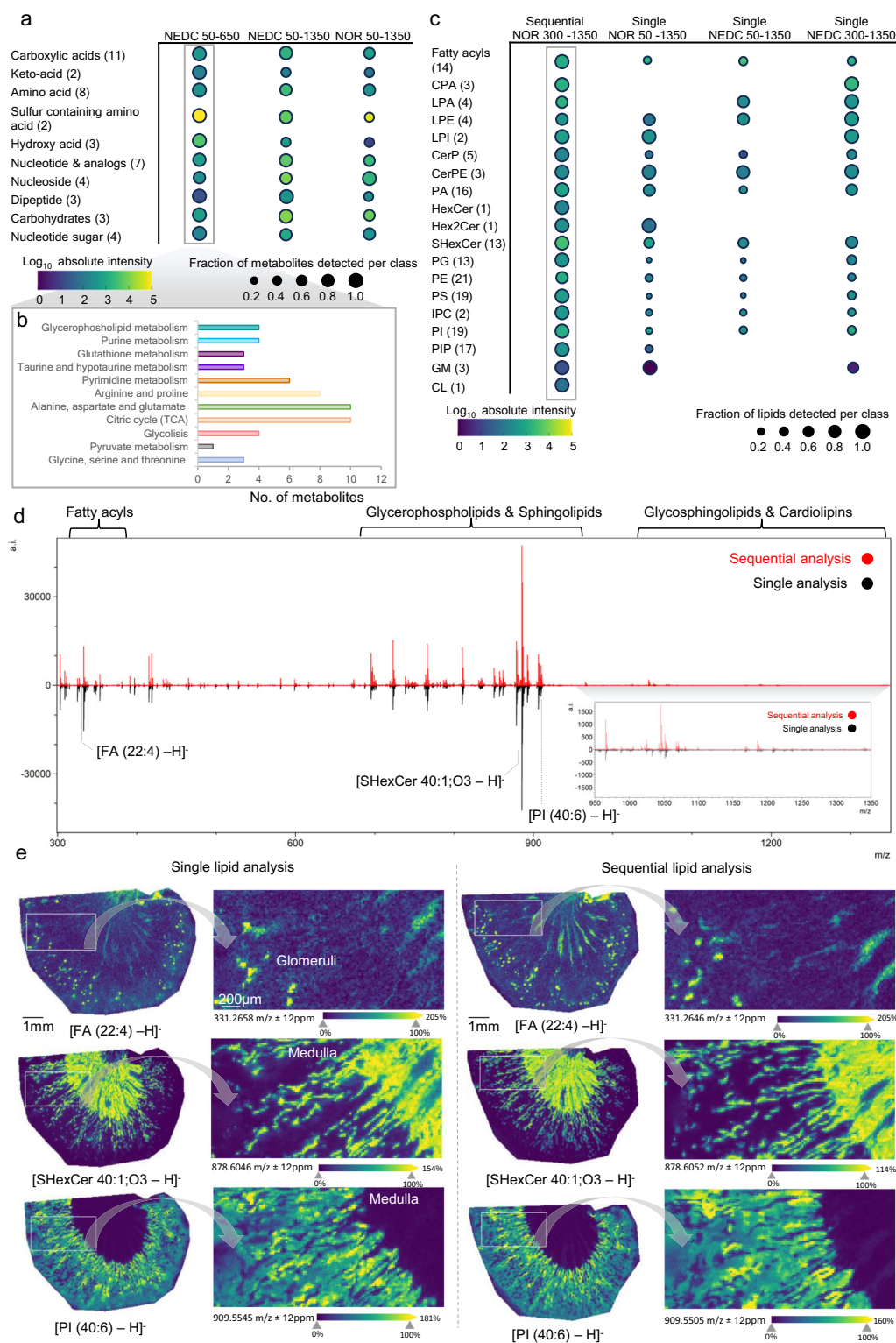


Fig. 2 | Metabolite and lipid coverage through single and sequential analysis.

a Detection of metabolites in chemical subclasses by different MALDI-MS matrices and detection ranges. The size of the dot represents the fraction of the metabolites detected per class amongst those considered. The color represents the average intensity of the detected analyte ions. The boxed area represents the optimal condition with the highest fraction of metabolites detected per class and average intensities. **b** Numbers of metabolites detected, with the optimal conditions, per metabolic pathway from the KEGG analysis. **c** Detection of lipids in chemical subclasses using different MALDI matrices and detection ranges. The size of the

represents the fraction of the lipids detected per class amongst those considered. The color represents the average intensity of the detected lipid ions. The boxed area represents the optimal conditions with the highest fraction of lipids detected per subclass and average intensities. **d** Spectra comparison between single (black) and sequential (red) lipid analysis. **e** Selected ion images of lipids from single or sequential analysis. *m/z* 331.262 [FA (22:4)-H]⁻ shows up in the glomeruli, *m/z* 878.604 [SHexCer (40:1;O3)-H]⁻ is found in the inner stripe of the medulla, and *m/z* 909.550 [PI(40:6)-H]⁻ is mainly in the outer stripe of the medulla and cortex.

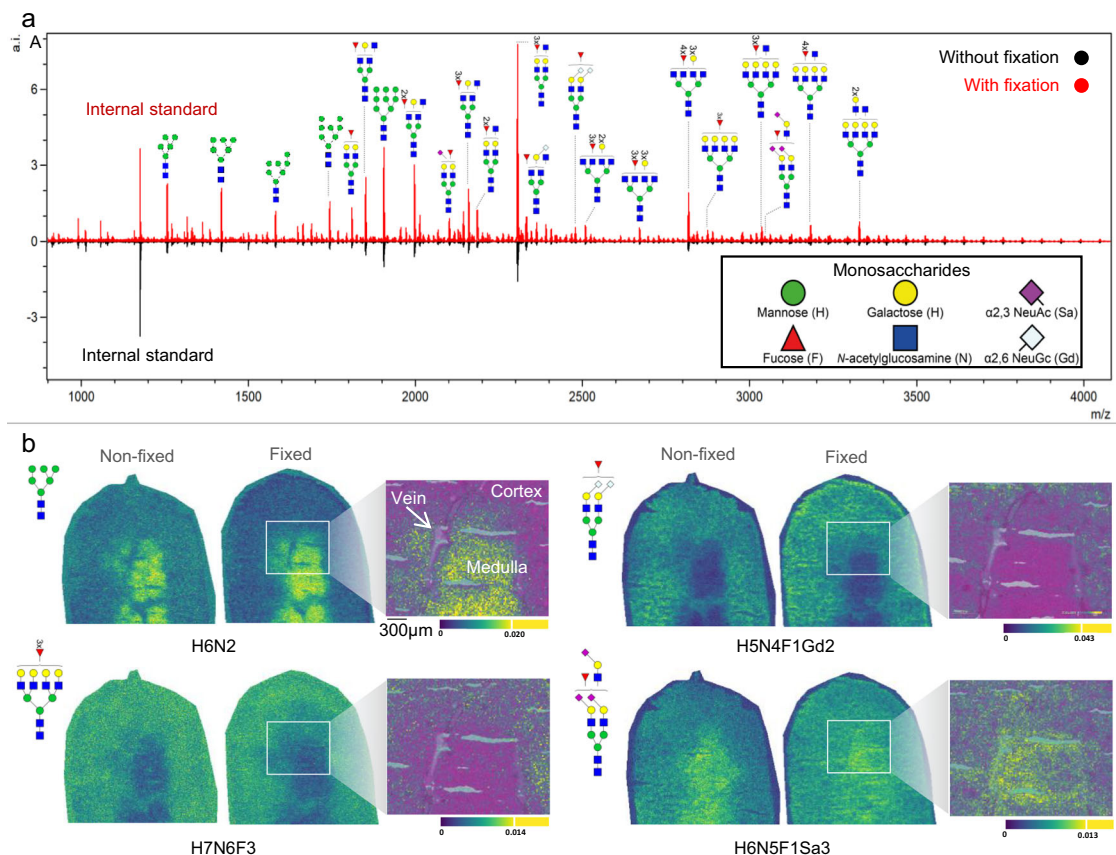


Fig. 3 | Optimized MALDI-MSI N-glycan analysis from fresh-frozen tissue.
a Mean MALDI-MSI N-glycan spectra of non-fixed (black) and fixed (red) kidney tissues with highlighted N-glycans. **b** Selected MSI ion images showing the distribution of N-glycans in medulla and cortex with and without on-tissue fixation. Image overlays of the fixed tissue section with optical images displaying the region

specific distribution of H6N2 (oligomannose), H7N6F3 (fucosylated complex type), H5N4F1Gd2 (α2,6-sialylated complex type) and H6N5F1Sa3 (α2,3-sialylated complex type). The selected ions were normalized to the total glycan count per pixel. The color bar represents a fraction of 1. H: Hexose, N: HexNAc, F: fucose Sa: α2,3-linked N-acetylneuraminic acid, Gd: α2,6-linked N-glycolylneuraminic acid.

Discussion

In this study, we present a fully integrated multi-omic MALDI-MSI approach for the spatial co-detection of metabolites, lipids, and N-glycans from a single fresh-frozen tissue section, providing a powerful platform for resolving complex molecular architectures within heterogeneous tissue microenvironments across multiple molecular classes. While we use murine kidney material as a challenging and heterogeneous model tissue, the method is readily applicable to a wide variety of mammalian tissue types. Importantly, the complete protocol is compatible with both fresh-frozen and frozen-embedded samples, though OCT-embedded material remains incompatible with metabolome analysis, due to matrix interference^{25,26}. A widely applicable alternative for OCT embedding is the use of hydroxypropyl methylcellulose/ polyvinylpyrrolidone (HPMC/PVP)-enriched hydrogel²⁷.

The depth of molecular coverage we obtained here, including the detection of sialylated glycans, with sialic acid linkage information and in a spatially preserved context, represents a significant advance compared to other sequential multi-omic MSI-based single tissue characterizations^{8,9,12}. On top of that, our advanced protocol enables a spatial resolution down to 20 μm, which is sufficient to differentiate molecular cellular phenotypes of the proximal and distal tubuli, glomeruli, ISOM/OSOM, collecting ducts, and blood vessels in murine kidney tissue, all tissue features with 20–100 μm fine structures. Higher spatial resolution, up to 5 μm, would allow single-cell characterization and is possible for individual metabolite and lipid analysis. However, as pixel size decreases (~5 μm), the sensitivity to detect metabolic features declines significantly, which necessitates the use of higher laser

energy, leading to tissue damage and reduced signal intensities in sequential analysis. While our protocol focuses on the metabolite and lipid analysis in negative ion mode, it does not exclude the option of performing analysis in positive or dual polarity using NOR as lipid matrix²⁸. However, while dual polarity imaging offers a more complete lipidomic profile¹², it limits the imaging area to every second pixel. This becomes critical when imaging highly heterogeneous tissues where the next pixel might already display a different cellular phenotype²⁹.

Isobaric/isomeric species pose a significant challenge in general MSI workflows where only MS1 data is considered. In our annotations for the metabolite and lipid features, we rely on accurate mass, accompanied, where possible, by collision cross section (CCS) values as obtained using trapped ion mobility spectrometry (TIMS). This narrowed the list of plausible lipid species per *m/z* feature but did not exclude all ambiguities (Supplementary Data 1). Further identification of highly correlated features was obtained by on-tissue MS/MS experiments on a consecutive tissue section.

For N-glycan feature annotation, accurate mass matching allows monosaccharide compositional assignments, although also here isomeric structures compromise full annotation. MS/MS annotations are required to distinguish between NeuGc-sialylated, fucosylated, and NeuAc-sialylated, afucosylated N-glycans, as well as to be able to assign structural features such as bisection, LacdiNAc epitopes, core and antenna fucosylation, and α-galactosylated species. In the current study, we rely on the comprehensive LC-MS/MS characterization of the murine kidney N-glycome as described by Helm and colleagues for glycan feature annotation³⁰. Yet, it is important to note that many

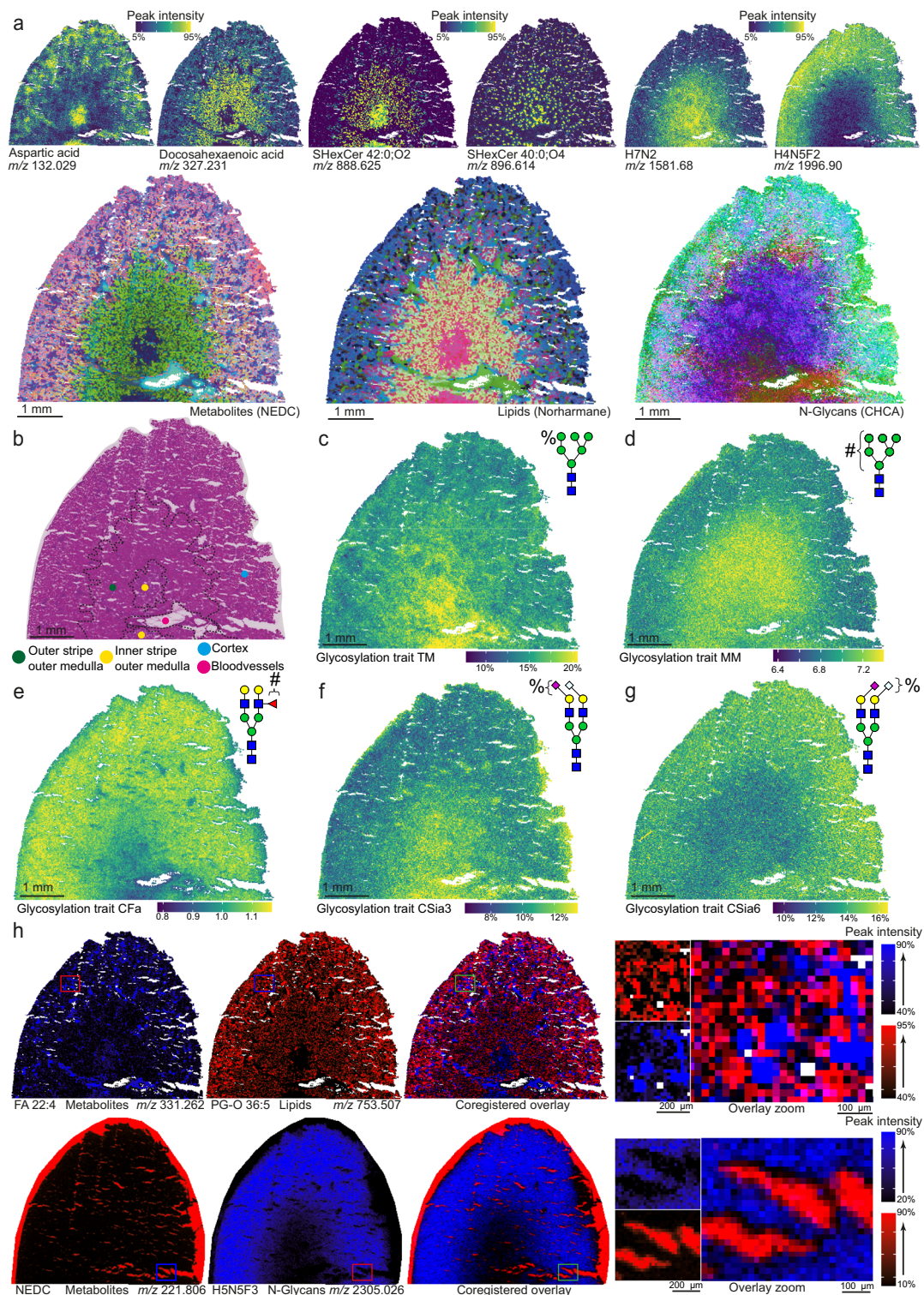


Fig. 4 | Sequential multi-omic MSI of a mouse kidney. Measurements of metabolites, lipids, and N-glycans were performed sequentially on the same tissue section. One of the three replicates is shown (the rest in Supplementary Figs. 7–10). **a** Corresponding single ion images of metabolites (m/z 132.029 aspartic acid, m/z 327.231 docosahexaenoic acid), lipids (m/z 888.625 SHexCer (42:2;O2), m/z 896.614 SHexCer 40:0;O4) and N-glycans (m/z 1581.68 H7N2, m/z 1996.90 H4N5F2) (top). The tissue heterogeneity captured in each omic layer is illustrated in a pseudocolor representation by reducing all heterogeneity to 3 dimensions with UMAP and scaling each dimension along either red, green, or blue (bottom). **b** Heterogeneity observed in the spatial omics measurements corresponds to distinct histological regions present in the kidney, mainly cortex, outer stripe of outer medulla (OSOM),

inner stripe of outer medulla (ISOM), and blood vessels. **c–g** Calculated glycosylation traits. **c** Oligomannose type N-glycans, as fraction of total N-glycans detected. **d** Average number of antennary fucoses of complex-type N-glycans, counting all fucoses beyond the first, which could be located on the core structure. **e** Average number of antennary fucoses of complex-type N-glycans combining NeuGc and NeuAc sialylated glycans. **f** α 2,3-Linked sialylated N-glycans as a fraction of all complex-type N-glycans combining NeuGc and NeuAc sialylated glycans. **g** α 2,6-Linked sialylated N-glycans as a fraction of all complex-type N-glycans combining NeuGc and NeuAc sialylated glycans. **h** Co-registered lipidomic and glycomic images using three anticorrelated co-registration points. Metabolite and lipid images were RMS normalized; glycan

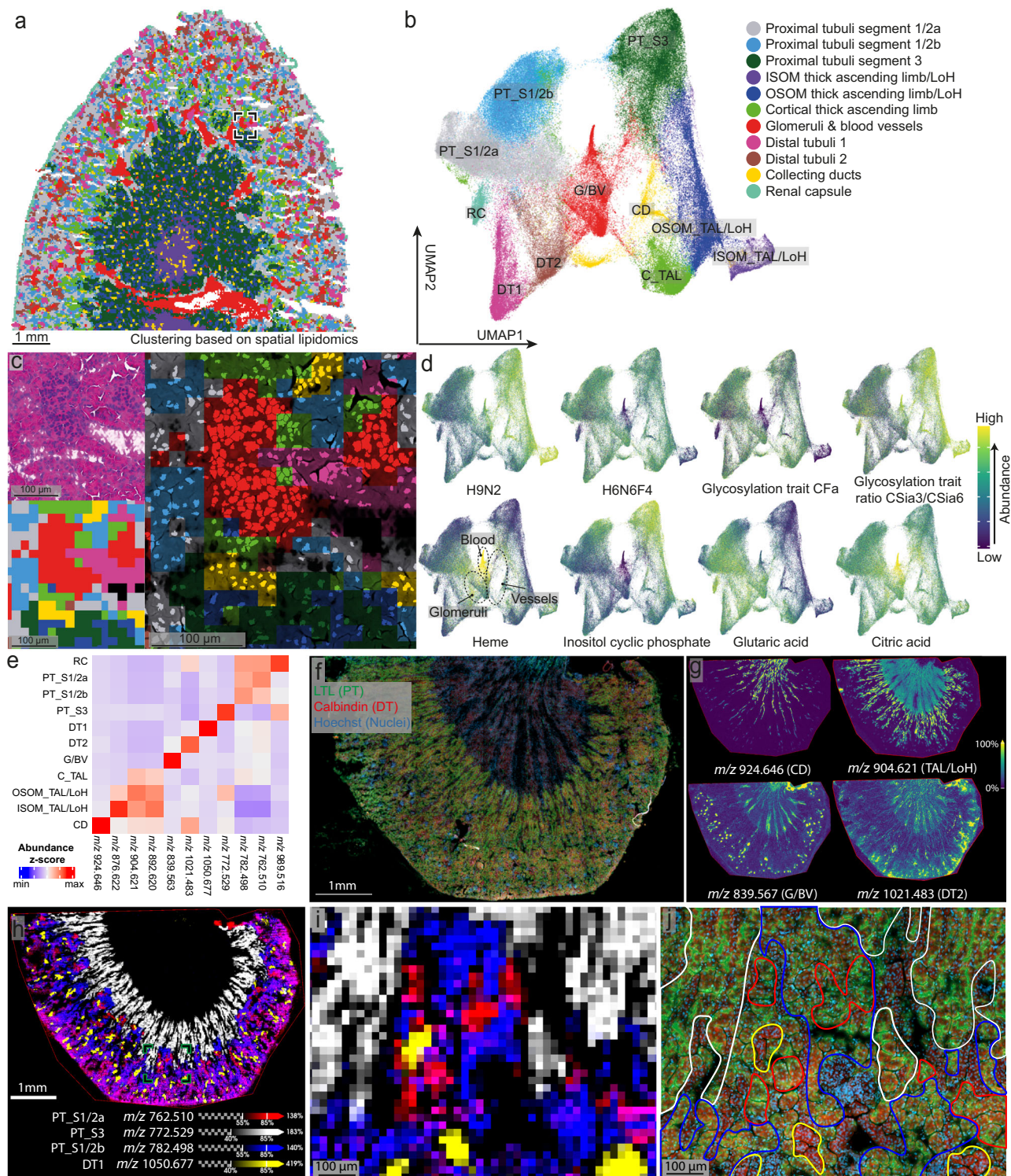
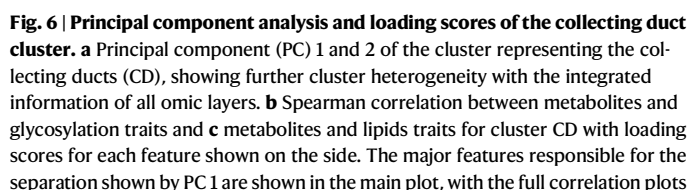


Fig. 5 | Clustering and integrated multi-omic analysis of the murine kidney. **a** Louvain clustering of the murine kidney based on all assigned lipids. **b** Cluster annotations displayed on a UMAP of all pixels with all assigned lipids as input. **c** Clusters co-registered to, and overlaid with, the histology picture, allowing cluster assignment. The image is zoomed in on a region with glomeruli in red (cluster G/BV) as highlighted in (a). **d** n-glycans, glycosylation traits, and metabolites can be projected onto the UMAP obtained from the lipid data and show specificity for sub-clusters. **e** Heatmap of signature lipids in different renal substructures as assigned in the kidney lipid atlas²³. **f** Immunofluorescence staining: LTL for proximal tubuli (PT, green), Calbindin for distal tubuli (DT, red), and Hoechst for nuclei (blue), post

MALDI-MSI to identify different renal substructures. **g** Specific lipid distributions of renal microstructures; *m/z* 924.646 (SHexCer 42:0;O4) in the collecting ducts, *m/z* 906.636 (SHexCer 42:1;O3) in OSOM thick ascending limb/LoH and ISOM thick ascending limb/LoH, *m/z* 839.567 (PG 38:1/LBPA 38:1) in the glomeruli and *m/z* 1021.483 in distal tubuli 2. **h** Overlay of signature lipids of the proximal and distal tubular segment; *m/z* 762.510 (PE 38:6) in PT_S1/2a, *m/z* 772.529 (PE O-40:8) in the PT_S3, *m/z* 782.509 (PS 36:4) in PT_S1/2b segment and 1050.677 in the DT1 segment. **i** Zoom into the overlay. **j** Co-registered immunohistochemistry image co-localizing different proximal tubular segments (red, blue, white) with LTL staining and the distal tubular segment DT1 (yellow) with Calbindin stain.



The kidney is a highly organized organ composed of multicellular functional tissue units. The cortex contains glomeruli and the proximal and distal convoluted tubuli, where blood filtration is initiated, and the majority of solute processing occurs. The medulla contains the loops

of Henle and collecting ducts, which generate and exploit osmotic gradients to concentrate urine. The renal papilla conveys the final urine into the minor and major calyces, which empty into the renal pelvis and subsequently the ureter^{40,41}. Through multi-omic integration, our objective is not only to distinguish these anatomical regions but also to elucidate how molecular features interact within specific functional units and microstructures.

Our pixel-to-pixel multi-omic integration enabled clustering analyses that incorporated all molecular layers simultaneously¹⁴. Among these, we found that lipid-based clustering alone yielded the highest level of spatial differentiation, clearly separating the glomeruli and blood vessels, ISOM_TAL, OSOM_TAL, CD, and enabling finer segmentation of PTs and DTs. This is expected, as lipid distributions closely reflect cell boundaries, whereas glycans and metabolites can also be secreted and occur within the extracellular matrix and tubular lumen. While we did not obtain the same level of tissue detail with each molecular class, combining metabolites and N-glycans with defined lipidomic clusters allowed their spatial patterns to be assigned to specific tissue microstructures. While this strategy does not fully overcome the intrinsic spatial resolution limitations of metabolite and glycan analyses, it leverages complementary information to infer biologically meaningful tissue organization beyond what any single modality can provide. For example, overlaying the lipid-based clusters with the data from the other modalities allowed further discrimination of e.g., the glomerular network and blood vessels, based on positive correlations between heme, tricarboxylic acid, α 2,3-linked sialic acids, and the pixels covering the blood vessels. This suggests that integrating multi-omic data is a valuable, but non-trivial task. A stepwise approach, sequentially combining data from individual molecular classes, may offer advantages over the direct integration of all variables in clustering analyses, as it leverages the strengths of each data layer.

The added value of stepwise data integration was further exemplified by the exploration of a lipid-based cluster that covers the CDs via an all-layer PCA within this cluster. The collecting duct represents the most distal functional segment of the nephron and is responsible for the final regulation of electrolyte composition, acid–base balance, and water reabsorption before urine enters the calyces and renal pelvis. It is composed primarily of principal cells, which regulate sodium, potassium, and water transport, and intercalated cells, which are specialized for acid–base homeostasis⁴². The PCA separated the CDs located in the ISOM and in the OSOM, and cortex, which could indicate the separation based on functionality of tissue structures. The features responsible for the highest variance in the first principal component correspond to sulfatides, which are highly correlated with taurine and metabolites involved in glycine, serine, and threonine metabolism. A recent mapping of the kidney lipidome suggested that several sulfatides (particularly SHexCer 42:2 and SHexCer 42:1) are markers of the thick ascending limb (TAL)²³, while SHexCer 40:0;O4 and, particularly, SHexCer 42:0;O4, are characteristic markers of collecting ducts in normal kidneys^{23,24}. These sulfatide species, containing phyto-sphingosine (t18:0) and 2-hydroxy FAs, were predominantly expressed in intercalated cells of CDs and have been speculated to regulate NH_3 and H^+ transport²⁴. Sulfatides play an important role in collecting ducts by forming an anionic layer on the plasma membrane, modulating membrane ion fluxes^{24,43,44}. Taurine and sulfatides, both involved in sulfur metabolism, functionally correlate in osmoregulation, membrane stability, and oxidative stress protection. Taurine transport is primarily regulated in cells of the loop of Henle and medullary collecting ducts as part of cell volume regulation mechanisms for cells to adapt to hyperosmolar conditions^{45–47}.

Furthermore, polyunsaturated glycerophospholipid (phosphatidylethanolamine; PE, phosphatidic acid; PA, phosphoinositides; PI and phosphatidylserine; PS) abundance shows a high correlation with α 2,6-sialylated N-glycans in the CDs located in the OSOM and cortex. These

phospholipids, containing poly-unsaturated fatty acids (PUFAs), have been shown to increase membrane fluidity⁴⁸, promoting protein mobility, while the α 2,6-linked sialic acids can be linked to apical protein sorting⁴⁹. PEs are major structural components of mitochondrial membranes and are essential for maintaining respiratory chain function and oxidative phosphorylation^{50,51}. Their high enrichment in CDs, previously reported by Farrow et al.²³, is consistent with the presence of mitochondria-rich intercalated cells to support ATP-dependent H^+ -ATPase activity⁵², and which show increased mRNA expression of Hk α ₁ and Hk α ₂ in cortical collecting ducts⁵³. This can also be functionally linked to tricarboxylic acid (TCA) cycle activity, which provides ATP necessary for ion transport processes, such as Na^+/K^+ -ATPase and H^+ -ATPase function in collecting duct cells^{53–55}. TCA cycle metabolites not only sustain cellular energy demands but may also act as signaling intermediates, coupling metabolic state to transport regulation within the collecting duct.

Although not all correlated molecules are biochemically linked, they appear to function together, or in parallel, to maintain osmoregulation, osmoprotection, antioxidant protection, ion transport regulation, and an anti-inflammatory effect in the CDs. Their co-expression likely reflects a shared protective strategy tailored to the physiological conditions of the nephron segments.

Our integrated omics MSI strategy demonstrates how a comprehensive molecular map can be constructed for highly complex and heterogeneous tissues. Larger, heterogeneous, cohorts with increased sparsity, batch effects, and biological variability may benefit from sparse generalized canonical correlation analysis, such as DIABLO⁵⁶, or probabilistic factor analysis frameworks, such as MEFISTO⁵⁷, to allow a more robust analysis of cross-modality relationships.

In summary, our multi-omics MSI approach reveals intricate correlations between molecular classes that define specific cell types and provides a foundation for generating hypotheses about the biochemical pathways active within them. By establishing this molecular framework, we lay the groundwork for deeper investigations into disease mechanisms, biomarker discovery, and the validation of therapeutic targets.

Methods

Ethical statements

The mouse tissue samples were obtained under the Leiden University Medical Center approved AVD project license number 11600202216547.

Samples

Kidneys were collected from control 6 to 8-week-old OF1 male mice (Charles River Laboratories) ($N=3$) and snap-frozen in liquid nitrogen. Consecutive tissue sections from the kidney ($N=25$, 3 biological replicates) were generated at 10 μm thickness using a cryostat and thaw-mounted on ITO slides. The tissue sections were freeze-dried for 20 min prior to matrix spraying.

Sample preparation for metabolites and lipids

For metabolite analysis, the samples were sprayed with 7 mg/ml NEDC (70% MeOH, 25% ACN, 5% MQ H_2O) using the HTX M3+ sprayer with the following settings: nozzle $T=60^\circ\text{C}$, 80 $\mu\text{L}/\text{min}$ flow rate, velocity 2000 mm/min, 3 mm track spacing, 20 layers, and 30 s drying time.

Lipid analysis was performed using norharmane matrix at 7 mg/ml in 90% MeOH and sprayed with the HTX M3+ sprayer with the following settings: nozzle $T=70^\circ\text{C}$, 110 $\mu\text{L}/\text{min}$, velocity 1200 mm/min, 3 mm track spacing, 15 layers, and 10 s drying time.

For the sequential analysis, the samples were first sprayed with NEDC matrix as described above, and following metabolite analysis, washed two times with 50 mM ammonium formate and dried in the vacuum desiccator. In the second step, norharmane matrix was sprayed for lipid analysis as described above.

Sample preparation for N-glycan imaging

For the N-glycan analysis providing sialic acid linkage-specific information, tissue sections were prepared as described previously by Holst et al.¹⁶. Briefly, tissues were washed in ethanol baths (100% ethanol, twice for 2 min), followed by water baths (twice for 5 min), and dried for 10 min in a vacuum desiccator. Prior to on-tissue derivatization, the samples were fixed in 10% formalin for 30 min. The tissues were washed with MiliQ two times and dried. On-tissue derivatization was performed by incubating the tissues slides in derivatization solution (250 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, 500 mM 1-hydroxybenzotriazole hydrate, and 250 mM dimethylamine in dimethylsulfoxide) for 1 h at 60 °C, followed by addition of a 25% ammonia solution (1:0.4 v/v derivatization solution-ammonia) and further incubation for 2 h at 60 °C, both protected from evaporation in a humid environment. After derivatization, tissue sections were rinsed thoroughly with 100% ethanol followed by sequential washes in 100% ethanol (2 × 2 min) and water (2 × 5 min). Slides were dried in a vacuum desiccator (10 min). On-tissue digestion was performed applying 8 layers at 30 µl/min of peptide N-acetyl-beta-glucosaminyl asparagine amidase (0.1 µg/µl in Ultrapure water; from N-Zyme Scientifics) using a HTX M3+ sprayer. N-glycans were released overnight at 37 °C in a humid environment. After incubation, slides were dried in a vacuum desiccator for 10 min, followed by the matrix application (5 mg/ml α -cyano-4-hydroxycinnamic acid in 50:49.9/0.1 (%v/v) ACN:mQ:TFA) co-sprayed with 5 µM maltoheptaose (in 50% ACN) using the HTX M3+ sprayer with the following settings: nozzle $T = 50$ °C, 80 µL/min, velocity 1200 mm/min, 3 mm track spacing and 6 layers.

MALDI-MSI analysis

The metabolite and lipid analysis were performed on the timsTOF flex MALDI-2 (Bruker Daltonics, Bremen, Germany) mass spectrometer equipped with a MALDI smartbeam 3D laser. The data were acquired beam scan mode, reaching $20\ \mu\text{m} \times 20\ \mu\text{m}$ spatial resolution, averaging over 150 laser shots per pixel. Different mass ranges were evaluated for combined metabolite and lipid analysis m/z 50–1500, metabolites m/z 50–650, and lipids m/z 300–1500. For metabolite m/z 50–650, the following settings were used: a transfer configuration of 50 V MALDI plate offset, –70 V deflection 1 delta, 125 Vpp Funnel 1 RF, 200 Vpp Funnel 2 RF, and 200 Vpp Multipole RF. Collision energy was set to 10 eV, with a collision RF of 350 Vpp. For lipid imaging, data were acquired with the scan range m/z 300–1500 and transfer configuration –70 V deflection 1 delta, 350 Vpp Funnel 1 RF, 350 Vpp Funnel 2 RF, and 300 Vpp Multipole RF. Collision energy was set to 10 eV, with a collision RF of 1800 Vpp. For combined metabolite and lipid analysis, the lipid ion transfer configuration was used. The N-glycan analysis was performed on the rapifleX MALDI-TOF/TOF-MS instrument (Bruker Daltonics) using a m/z range of 900–3300 at $20\ \mu\text{m} \times 20\ \mu\text{m}$ spatial resolution, averaging 150 shots per pixel. The metabolite and lipid data were collected in negative ion modes while the N-glycan data were collected in positive ion mode. N-glycan accurate mass analysis on the timsTOF flex was performed using a m/z range of 900–3700. Data were acquired at $20\ \mu\text{m} \times 20\ \mu\text{m}$ spatial resolution, averaging 200 shots per pixel. The following ion optics were used: 50 V MALDI plate offset, 90 V deflection 1 Delta, 500 Vpp Funnel 1 RF, 500 Vpp Funnel 2 RF, and 500 Vpp Multiple RF. Collision energy was set to 25 eV with a collision RF of 4000 Vpp. Transfer time for the TOF was set to 110 µs with 27 µs pre pulse storage.

After MSI analysis, residual MALDI matrix was removed with a series of washes with ethanol, and tissues were stained with hematoxylin and eosin (H&E) following routine histopathological procedures. Stained tissue sections were scanned with a high-resolution ZEISS Axio Scan.Z1 digital slide scanner, and the resulting images were exported to a .TIFF format. Tissue structures were annotated through histological analysis by an expert pathologist (J.K.).

Ion mobility and MS/MS analysis

The putatively assigned metabolites and lipids were further curated based on collisional cross-section (CCS) values. For CCS-enhanced identification of m/z features, spectra were recorded in timsON mode at a $20 \times 20\ \mu\text{m}^2$ spatial resolution covering a m/z ranges between 50–650 and 300–1500 and summing 150 laser shots. Ion mobility separation was achieved over a 300 ms ramp over a $1/K_0$ interval ranging from 0.6 to 1.6 V s/cm². MS/MS analysis was performed on highly correlated metabolites and lipid ions with the precursor ion selection window of 1 Da and varying collision energy between 20 and 50 eV.

Data preprocessing

The metabolite and lipid data were imported into SciLS Lab 2025b Pro version (Bruker, Daltonics). The overview spectra were exported in csv format, and the spectra were imported into open-source mMass software (<https://github.com/xxao/mMass>). The mean spectrum was re-calibrated using the imported peak list from endogenous compounds present in the tissue using a quadratic equation. The spectra were subjected to peak picking with an absolute signal threshold of 3 and the deisotoping setting activated. The peak lists were then imported back into SciLS Lab for further processing. Matrix peaks were removed from the peak list, and accurate mass identification was performed through HMDB⁵⁸, Metaspace⁵⁹, and LIPID MAPS^{60,61} libraries. Raw data files were loaded into SciLS Lab 2025b Pro version (Bruker, Daltonics). Feature selection was performed in the timsON dataset by using the T-ReX³ feature-finding algorithm in SciLS Lab (parameters: neighborhood size – 4×4 , coverage – 100%, relative intensity threshold – 0.3%). Lipid annotations were performed based on accurate mass against the Lipid Maps Structural Database using the bulk structure search (parameters: [M-H][–] and [M+Cl][–] ion adducts and ± 0.05 mass tolerance). The molecular annotations with the mass error tolerance below 5 ppm were kept for further analysis. The query masses (Da) and collision cross section ($\text{\AA}^2$) were compared to the theoretically predicted CCS values in the Human Metabolome Database (HMDB) (parameters: ± 0.05 molecular weight tolerance, AllCCS prediction method, and 5% CCS error tolerance). Subsequently, the annotated features were manually curated, taking possible molecular ions in negative mode, low abundant lipid species, as well as potential in-source fragments into account. Remaining ambiguities in annotations are indicated in Supplementary Data 1.

The N-glycan data were imported into SciLS Lab 2024b Pro version using the Top Hat baseline removal algorithm. The data were normalized to the total N-glycan intensity per pixel. The overview spectra per tissue were exported into csv file for manual peak picking of mono-isotopic peaks. Sodiated N-glycan compositions were assigned in GlycoWorkbench (version 2.1) through the Glyco-Peakfinder tool. Assignments were further curated based on ppm error of the data acquired on the timsTOF flex (<10 ppm), isotope scores as calculated in MassyTools after re-calibrated using an imported peak list from endogenous compounds present in the tissue, common knowledge of naturally occurring glycan structures, as well as literature on murine kidney N-glycans³⁰ (Supplementary Data 2). Gly-TouCanIDs were assigned to each glycan identification⁶². Hexose, HexNAc, and fucose monosaccharide subunits of N-glycans were abbreviated to H, N, and F, respectively. For sialic acids, $\alpha 2,3$ -linked *N*-acetylneuraminic acid and $\alpha 2,6$ -linked *N*-acetylneuraminic acid were abbreviated to Sa and Sd, whereas $\alpha 2,3$ -linked *N*-glycolylneuraminic acid and $\alpha 2,6$ -linked *N*-glycolylneuraminic acid were abbreviated to Ga and Gd. Further curation was based on a previously described comprehensive analysis of the murine kidney N-glycome³⁰. Both *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid were described to be present in the murine kidney, as well as high levels of antennary fucosylation. Oligo- or poly-sialic acids were not described, neither was $\alpha 1,3$ -galactosylation. For this reason, compositions with antennary fucosylation were favored over oligosialylated compositions. Finally,

LacdiNAc and fucosylated LacdiNAc groups are also expected in the murine kidney, which was taken along during the curation. Finally, for isobaric assignments, such as H6N4Sd2F2 and H5N4Sd1F3Gd1, the raw LC-MS/MS data from Helm and colleagues³⁰ were loaded into Skyline⁶³ and manually inspected for the presence of diagnostic fragments for *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid. In cases where both fragments were present, no further distinction was made. The final glycan compositions and structural features were assigned to the best of our knowledge based on accurate mass, literature, and *N*-glycan MS/MS data of murine kidney material. Except for sialic acids, monosaccharide linkages were not determined. Ambiguities in annotation are indicated in Supplementary Data 2.

For each pixel, glycosylation traits TM, MM, CSia3, CSia6, CF, CFa, and CLN were calculated (Supplementary Data 3). These are measures for the oligomannose proportion of total, oligomannose size, α 2,3-linked sialylated proportion of complex type *N*-glycans, α 2,6-linked sialylated proportion of complex type *N*-glycans, degree of fucosylation, degree of antennary fucosylation, and branching or number of LacNAc groups, respectively. Of the 71 curated *N*-glycans used for glycosylation trait calculations, 8 were a mixture of isobaric/isomeric species (Supplementary Data 2). These species, such as H6N4Sd2F2 and H5N4Sd1F3Gd1, could, for example, count for 1 or 2 antennary fucoses in the CFa calculation. To detail the minimum and maximum effect of these ambiguous annotations, the glycosylation traits for CF, CFa and CLN were calculated using either the highest or lowest possible values in the calculations, showing no meaningful effect on the distribution of these traits (Supplementary Fig. 3 and Supplementary Data 3).

Data integration and analysis

Metabolite ion images were co-registered to reference ion images from the lipidomic and *N*-glycomic datasets in SCiLS. The co-registered metabolite ion image served as a common reference linking the lipidomic and *N*-glycomic datasets to the metabolic dataset, enabling spatial alignment and multimodal integration through the SCiLS Ion Image Mapper. The pixel-level integrated intensity values were exported to csv files with in-house R scripts utilizing the SCiLS API plugin. UMAP dimensionality reduction, Louvain clustering, and histology overlay with spatial clustering were essentially performed as described⁶⁴. Briefly, UMAP and Louvain clustering was performed with the Seurat package (v 5.3) in the R programming language (v 4.3.2). The obtained clusters were overlaid with histology by loading the histological scan with EBImage (v 4.36.0), and affine coregistration of a low-dimensional representation of the data to histology was done with the Rniftyreg package (v 2.8.4). This same affine transformation was applied to the spatial cluster image.

Inter-omic Spearman correlations and PCA were calculated following feature centering according to the mean and scaling to the standard deviation for the curated datasets. Results were visualized with the ComplexHeatmap (v 2.10.0) package.

Co-registration accuracy assessment

Co-registration accuracy between every modality was assessed. Taking a single peak for each modality to compare, one was selected as the fixed image and one as the moving image. Translation of the moving image was done in the range of -1 to $+1$ in both the *x* and *y* directions, in combination with rotation between -1° and $+1^\circ$ (in steps of 0.1°). The signal from both images was discretized with varying numbers of bins (between 20 and 50, in steps of 10). Normalized mutual information between the fixed and moving image was calculated with the “theoinfo” package (v 1.2.0.1) for each of the shifts and compared to the original co-registration in the R programming language.

Immunofluorescence staining

After MALDI-MSI of metabolites and lipids, the MALDI matrix was removed using an ethanol gradient. Slides were then blocked for 30 min with serum-free protein block (Dako), followed by an additional 45-min blocking step at room temperature using mouse-on-mouse blocking reagent (Vector Laboratories). Sections were incubated overnight at 4°C with calmodulin primary antibody (CB-955, 1:400, Merck). The following day, slides were incubated for 1 h at room temperature with goat anti-mouse IgG AF568 secondary antibody (1:300, Invitrogen, A21124). After an additional 10-min blocking step with serum-free protein block (Dako), sections were incubated overnight at 4°C with *Lotus tetragonolobus* lectin (LTL, 1:300, Vector Laboratories). The next morning, slides were incubated for 1 h at room temperature with streptavidin-AF750 (1:300, Invitrogen, S21384). Finally, tissue sections were incubated with Hoechst 33258 (1:5000, Invitrogen, H3569) for 30 min at room temperature and mounted with ProLong Glass Antifade Mountant (Invitrogen, P36984). Fluorescent images were acquired using the ZEISS Axio Scan.Z1 digital slide scanner, exported as TIFF files, and co-registered with the MALDI-MSI data.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Unless otherwise stated, all data supporting the results of the study can be found in the article, Supplementary Data, and Source data files. The MALDI-MSI data generated for the study has been converted to imzML files and were deposited on METASPACE repository under accession code [<https://metaspace2020.org/project/ogrinc-2025>]. Due to size limitations of open repositories, all raw MSI data in .mis format, including single metabolomics, lipidomics, *N*-glycomics, sequential lipidomics, *N*-glycomics, ion mobility, and MS/MS data, are available upon request. Source data are provided with this paper. Source data are provided as a Source data file. The Source data xls file contains numerical data for Figs. 2a–d, 3a, 5e, 6a–c and Supplementary Figs. 1, 2, 4, 5, 11, 13–15. Source data are provided with this paper.

Code availability

The R code for multiomic integration accompanying the manuscript is available on Code Ocean [<https://codeocean.com/capsule/6204031>].

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Author contributions

Conceptualization and funding were carried out by N.O., M.W., and N.H. Experiments were performed by N.O., R.U., and H.D. Data analysis was done by N.O., R.U., and H.D.; data interpretation by N.O., R.U., and N.H. N.O., R.U., and N.H. wrote the manuscript. Histopathological annotations were performed by J.K. The study was supervised by M.W. and N.H. Reviewing and editing was carried out by N.O., J.K., M.G., M.W., and N.H. All authors have approved the final version of the manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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