PIA SSN: Parallel Image Acquisition and Spatial Similarity Network for Tandem Mass Spectrometry Imaging

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Abstract

Unambiguous molecular annotation in mass spectrometry imaging is essential for understanding complex biochemical processes. Here, we present Parallel Image Acquisition (PIA), for simultaneous imaging with high mass resolution and tandem mass spectrometry, and Spatial Similarity Network (SSN), to annotate isobars and isomers and reveal molecular colocalization. With PIA SSN we uncover phospholipid isomers and isobars in mouse brain tissue and derive cholesterol oxidation metabolites linked to multiple sclerosis.

Main

Mass spectrometry imaging (MSI) enables label-free visualization of molecular distributions in biological tissues, making it a central technique for spatial lipidomics and metabolomics.[1] While high mass resolving power and ion mobility spectrometry enhance molecular specificity, tandem mass spectrometry (MS^2) is required to distinguish isomers and unresolved isobars.[2,3] Consequently, imaging using tandem mass spectrometry (MS^2I) is essential to unravel the distributions and biological roles of isomers and isobars.[4,5 6] Despite its advantages, MS^2I is rarely performed due to challenges in sensitivity, maintaining spatial resolution and throughput, and ensuring repeated precursor selection for MS^2 in each pixel, which is crucial for generating product ion images. Current MS^2 approaches, such as data-dependent acquisition (DDA),[7] dataset-dependent acquisition,[8] and conventional targeted MS^2 imaging[9] fail to address these limitations. Therefore, a new strategy for accurate molecular annotation in MSI is needed.

We present a novel MS^2I strategy that overcomes previous technical challenges by employing Parallel Image Acquisition (PIA). To reveal colocalizing ions and annotate hidden isomers and isobars we pair this with a new concept of correlating ion distributions with a Spatial Similarity Network (SSN) tool. To enable PIA, we integrated pneumatically assisted nanospray desorption electrospray ionisation[10] (PA nano-DESI) with a tribrid mass spectrometer consisting of a quadrupole, an Orbitrap (FT), and an ion trap (IT) mass analyzer and set up a method for targeted MS^2 in the IT during the acquisition of a high resolution ($m/\Delta m$ = 500,000 at m/z 200) scan in the FT (Fig. 1a). Notably, we achieved up to 28 $ITMS^2$ scans during one FT scan when imaging mouse brain tissue sections (Fig. 1, S1-2). Consequently, each pixel of the acquired ion image contained one high mass resolution spectrum and 28 MS^2 spectra without compromising spatial resolution or throughput (Fig. S2). We further extended this by looping N=4 inclusion lists of 27 precursors to gain 108 MS^2I with four times larger pixels (Fig. 1a-e, Table S1). The vast amount of complex data generated motivated us to develop the new SSN tool that clusters ions by their distributions. The SSN tool, which is accessible within our ion-to-image (i2i) software (Fig. E1),[11] visualizes computed spatial similarity scores of either MS^2I or MS^1I images as a network (Fig. 1g, E2-4, Fig. S3 and S4). Furthermore, by adding a library, the tool enables automatic annotation of correlated product ions for subsequent cross-referencing with accurate mass derived putative precursor ion annotations (Fig. 1h-i, E4, S5 and Table S2-4). Notably, in 34 of the 108 $ITMS^2$ scans we visualized and annotated 134 isomers and isobars consisting of 70 phosphatidylcholine (PC), 44 phosphatidylethanolamine (PE), and 20 PE-plasmalogen (Fig. E5, S6, and Table S5-6). Overall, with PIA SSN, product ion distributions are acquired and used as a third orthogonal dimension alongside accurate mass measurements and fragmentation spectra for unambiguous annotation of otherwise non-resolvable ions.

The PIA SSN workflow enables annotation beyond accurate mass, which is necessary to gain biological insights. We applied PIA SSN in a pilot study targeting cholesterol and oxidized cholesterol products. Cholesterol is a main constituent of myelin sheets and, therefore, of interest in demyelination, which is the pathological hallmark of multiple sclerosis.[12–15] By employing silver-doped PA nano-DESI MS^2I, we successfully imaged cholesterol and six

oxysterols in human white matter brain tissue (Fig. 2, S7-9). The $ITMS^2$ isolation windows captured multiple precursors, but the SSN efficiently clustered and annotated the diagnostic product ions (DPIs) (Fig. 2a-d, E6, S8, Table 7).[16] For example, the DPIs of cholestanetetraol (ST6) and 24-homodeoxycholic acid (ST7) reveal their distinct localization to a blood-brain barrier (BBB) leakage site, which is highly relevant to the known increased BBB leakage in multiple sclerosis lesions (Fig. 2e).[17] Subsequently, we conducted a multimodal study to investigate cholesterol oxidation in demyelination. First, we used $FTMS^2I$ to confirm the exclusive presence of cholesterol and the three oxysterols ST3, ST4 and ST7 in their accurate mass channels (Fig. S10). Then, proteolipid protein (PLP) expression and quantitative $FTMS^1I$ images were combined from three non-neurological controls (NNC) and five people with multiple sclerosis (PwMS) (Fig S11). Specifically, regions of interest were categorized by PLP as normal, diffuse, or absent and transferred to the ion images for data extraction and analysis (Fig. E7). We report a more than 2-fold depletion of cholesterol, a ~2-fold increase of the oxysterols ST4 and ST7, and an overall increased oxidative environment in PwMS brain tissue (Fig. 2f-g and E8). Additionally, the ST3, that has been previously reported to be neurotoxic, shows an increasing trend.[18] Our results support the increased BBB leakage in lesions and indicate that distinct oxidized cholesterol species can play a previously unknown role in lesion formation in multiple sclerosis that necessitates further investigation.

In summary, PIA SSN facilitates high-precision spatial omics with extensive metabolite coverage by combining high mass resolution MS^1I and MS^2I. Furthermore, it enables molecular annotation based on three dimensions of data: accurate mass, product ion spectra, and spatial similarity, all neatly packaged within our i2i software. Using our workflow, we report characterization of isomer/isobar-specific phospholipid distributions in mouse brain tissue. Furthermore, we present the first-ever oxysterol profiling in human multiple sclerosis brain tissue, strongly indicating cholesterol oxidation as part of the pathological hallmark. We acknowledge that coupling our method with higher spatial resolution ion sources and reactive chemistry workflows could lead to even deeper structural characterization.[19,20] Ultimately, PIA SSN pioneers the integration of spatial omics with MS^2I, setting a new standard in spatial lipidomics and metabolomics.

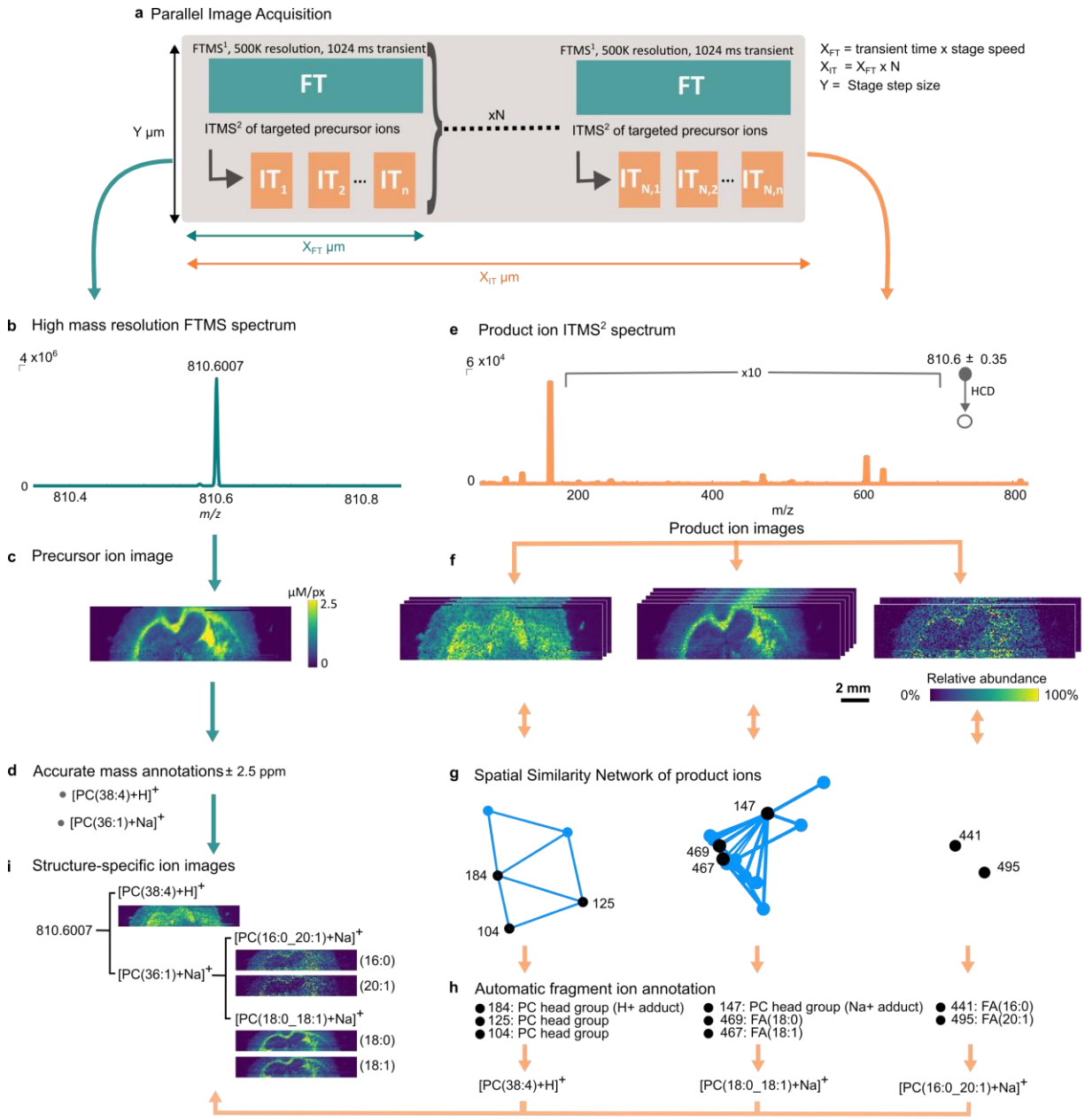

Fig 1 | Workflow of PIA SSN. a, Schematic of PIA where one full FTMS scan with n serial ITMS^2 scans are acquired in each pixel. Additional precursors are included by looping N number of inclusion lists. **b,c,d,** The acquired FTMS spectrum from mouse brain tissue has high mass resolution **(b),** quantitative MS^{1}I is achieved through normalization to internal standards in the PA nano-DESI solvent **(c),** and putative annotation of the precursor is enabled by accurate mass **(d)**. In the example FTMS spectrum **(b),** putative annotation *m/z* 810.6007 suggests [PC(38:4)+H]$^+$ and [PC(36:1)+Na]$^+$. **e,f,g,h,** All product ions from all precursors in the isolation window are detected in the ITMS^2 spectra **(e)** and each have their unique product ion image **(f)**. Product ion in this example **(e)**, were grouped based on their spatial similarity score **(g)**, calculated by the SSN addition to our i2i software. Product ions are annotated through our in-house compiled library **(h)** that in combination with the accurate mass annotation enables full structural annotation of *m/z* 810.6007 revealing an **(i)** isobaric mixture of [PC(38:4)+H]$^+$ and [PC(36:1)+Na]$^+$ and an isomeric mixture of [PC(18:0_18:1)+Na]$^+$ and [PC(16:0_20:1)+Na]$^+$. HCD, higher energy collision-induced dissociation. N=4 and n=27.

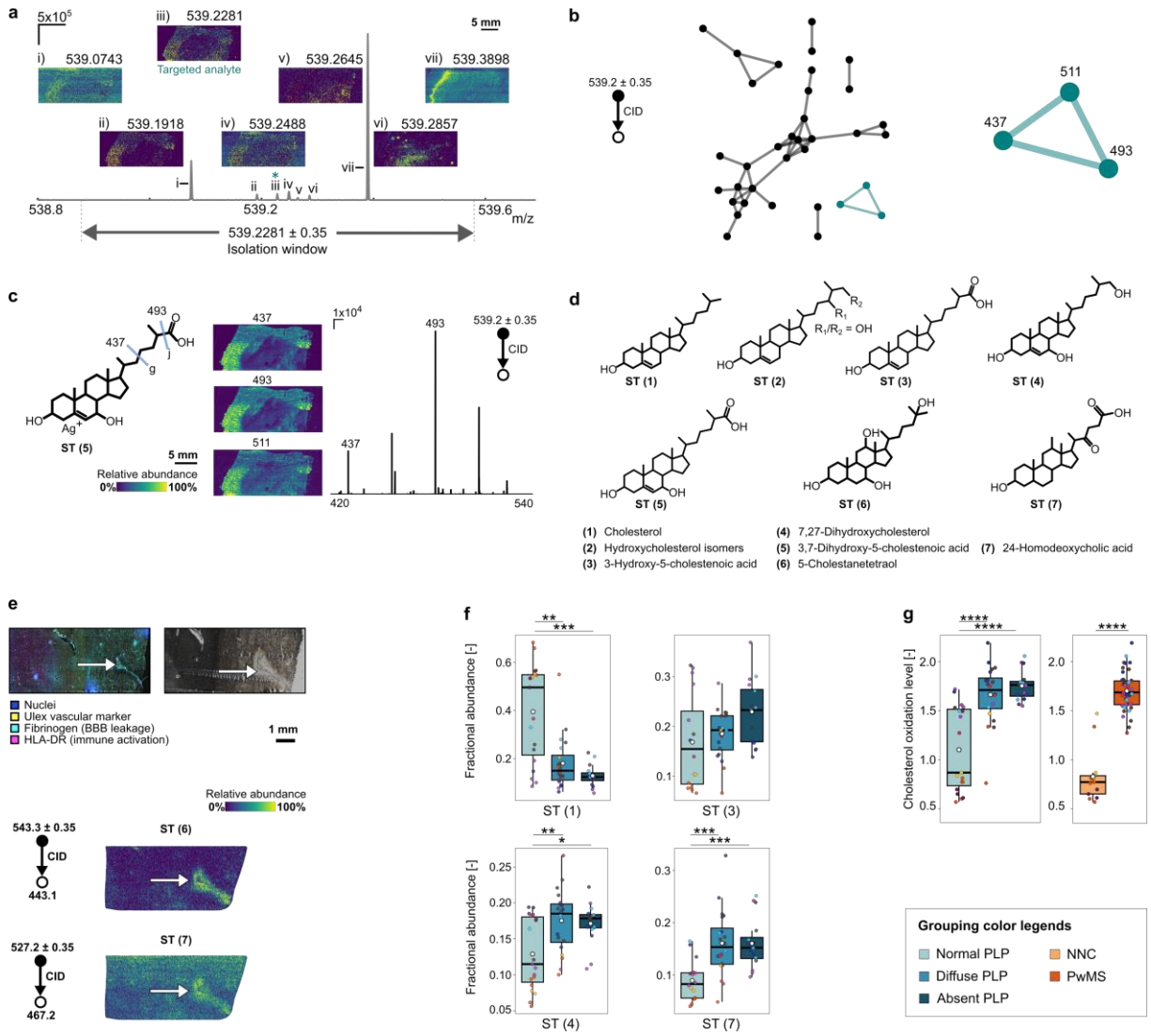

Fig 2 | Cholesterol oxidation products detected in human multiple sclerosis brain tissues. a,b,c,d, Structural annotation of cholesterol and oxysterols $[M+^{107}Ag]^+$. An FTMS spectrum detailing the isolation window of ions selected for $ITMS^2$ **(a)** with the corresponding SSN clusters **(b)** and an example (*m/z* 539.2) with product ion images and fragmentation sites from one cluster **(c)**. Structure of cholesterol and identified oxysterol species from tissue **(d)**. **e,f,g,** Multimodal study revealing altered cholesterol and oxysterol profiles in demyelinated areas. Representative image of UEA-I, Fibrinogen and HLA-DR immunoreactivity and optical image with corresponding images of diagnostic product ions for cholestanetetraol (ST 6) and 24-homodeoxycholic acid (ST 7), white arrow: BBB leakage site **(e)**. Boxplots represent the fractional abundance of significantly decreased cholesterol (ST 1) and increased oxysterol (ST 3, ST 4, ST 7) species **(f)**. Point colors indicate individual donors as per Table S8, black line denotes the median and the white point the mean **(g)**. Level of oxysterol oxidation significantly increases in diffuse and absent PLP category compared to normal PLP and is higher in PwMS compared to NNCs. For calculations see Methods section. PLP, proteolipidprotein staining of myelin; HLA-DR, Human Leukocyte Antigen – DR isotype; BBB, blood brain barrier; Ulex: Ulex Europaeus Agglutinin I (UEA I). (*:p<0.05, **:p<0.01, ***:p<0.001, ****:p<0.0001).

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Methods

Sample selection for model experiments

Mouse brain (8–10 weeks old male C57BL/6) was purchased from Creative Biolabs (Shirley, NY, USA) and cut to a thickness of 10 µm using a cryo-microtome (Leica Microsystems, Wetzlar, Germany). The sections were thaw-mounted onto regular microscope glass slides and stored at -80 °C prior to analysis.

Sample selection for analysis of human tissue samples

Freshly frozen post-mortem human brain samples were obtained from the Netherlands Brain Bank from 5 clinically diagnosed MS patients (mean age at death: 65 years) and 3 non-neurological controls (mean age at death: 66 years). Tissue blocks were cut into 10 µm sections and stored at -80 °C until further use. All donors or their next of kin provided fully informed consent for autopsy and use of material for research from the Netherlands Brain Bank under ethical approval by the Medical Ethics Committee of the Free University Medical Center in Amsterdam (2009/148), project number 1,127. Donor details are shown in Table S8.

Parallel image acquisition using PA nano-DESI

Samples were gently thawed at room temperature under constant airflow before analysis. Mouse brain sections were analyzed without any sample preparation, human multiple sclerosis tissue sections were dipped six times in deionized water (Milli-Q, 18.2 MΩ) for one minute each to remove excess salts. For MSI, the samples were placed on an XYZ linear motor stage (Zaber Technologies Inc., Vancouver, BC), controlled via a custom-designed LABVIEW program.[21]

The PA nano-DESI was set up as previously described, [10] using 150/50 µm o.d./i.d. fused silica capillaries (Genetek, Sweden). Briefly, the primary capillary, connected to a syringe containing the nano-DESI solvent, was coupled to a pneumatically assisted secondary capillary positioned at ~90 degrees. The extraction solvent was delivered through the primary capillary via a syringe pump (Legato 180, KD Scientific, Holliston, USA), with the ESI voltage applied directly to the syringe needle. The probe and sample positioning were monitored using long-working-distance digital optical microscopes (Dino-Lite, USA).

Mass spectrometry was performed in positive ion mode on an Orbitrap IQ-X (Thermo Fisher Scientific, San Jose, CA, USA) using high-resolution Orbitrap (FTMS) MS^1 and ion trap (ITMS) MS^2 modes, or $FTMS^2$ for accurate mass confirmation. For PIA, ions were first selected for a full FTMS scan and subsequently for several parallel MS^2 scans in the ion trap during the FTMS transition time. The PIA-SSN pipeline was tested across five different experimental conditions (varying extraction solvents and targeted analytes) to demonstrate its versatility (Table S6). Specific PA nano-DESI and mass spectrometry acquisition parameters for each experiment are provided in Table S9.

Figures showing data and metadata from the experiments was exported from FreeStyle 1.8 SP2, v1.8.63.0. Ion images were generated using our in-house software tool, i2i,[11] from .mzML data converted via MSConvertGUI (ProteoWizard, v3.0.22285) from .RAW files. The use of .mzML format facilitates a vendor-neutral use of the software. Ion images of the MS^2 data was created by imputing the scans on a master grid generated from the FTMS scans where the specific MS^2 spectra were given the same coordinates as the closest FTMS master scan.

Spatial similarity network analysis

The SSN is computed based on intensities of all identified *m/z* values in all pixels. First, the user selects the scan filter and scan type, and the parameters. Although the GUI provides suggested pre-set parameters for ppm tolerance, and min and max intensities, these may need to be altered based on the experiment. Secondly, the user selects if the SSN should be computed using either the sum squared error (SSE) or the cosine distance metric.

When the user clicks the run button, a non-targeted search, based on the user-defined parameters, is conducted to identify *m/z* values that will be considered in the SSN. The algorithm for the search is identical to the algorithm in the non-targeted tab of the i2i.[11] Following, one of the *m/z* values is selected as an *anchor ion image* and the distance metric is calculated for all identified *m/z* values against the anchor ion image. To *normalize* the response of different ions, all compared *m/z* values are first scaled to the 99th percentile of intensity in the respective ion image. This enables all *m/z* values to be compared despite varying absolute intensities. Subsequently, the anchor ion image is exchanged to ensure that all ion images of identified *m/z* values are compared with each other.

The equation used to calculate the SSN based on SSE and cosine distance is shown in Eq. S1 and Eq. S2, respectively. The x and y are intensities of the respective *m/z* in a given pixel, the x_{anchor} is the matrix of the anchor ion image and y_i is the matrix of the ion image for the *i*th ion.

$$SSE = \sum |x_{anchor} - y_i|^2 \qquad \textit{Eq. S1}$$

$$Cosine\ distance(x_{anchor}, y_i) = 1 - \frac{x_{anchor} \cdot y_i}{\|x_{anchor}\| \|y_i\|} \qquad \textit{Eq. S2}$$

The computational process creates a lower triangular correlation matrix with values corresponding to a similarity index (SI). The use of a difference matrix saves memory usage and computation time. The ion images with high SI, as set by the user as the error limit, are grouped based on the user selected percentile of the similarity index values to be included. For example, by selecting 0.1, only the 10% of the lowest SI values (i.e. the SI values with the highest correlation) will be included in the filtered difference matrix. The error limit has to be optimized for each experiment with respect to the complexity of the given scan event. A graph in MATLAB is then created with the filtered difference matrix, with each group in the difference matrix corresponding to a connected component of the graph. Following, the function conncomp is used to extract the spatially correlated *m/z* values of the groups. The results are shown as clusters of *m/z* values with similar distributions, termed the SSN.

To interrogate the SSN, the user can select groups and their *m/z* values in the list box next to the displayed SSN. Alternatively, by opening the SSN as a plotted figure, the user can hover over the displayed SSN to view information on each node in real time and save the plot. The number of the individual SSN groups are ordered by size, where Group 1 is the largest group. The graph layout, including the length of the lines within each SSN group is automatically set by MATLAB and has no value for the interpretation of the SSN. For automatic annotation of the *m/z* values in the SSN based on user selected ppm difference, the user can upload a database containing *m/z* values and known names of the corresponding ions. The annotated ions are identified in the list box by a green dot next to the *m/z* value. All SSN calculations and interrogation are supported by a GUI integrated in our i2i application[11] (github: LanekoffLab), and the functionalities are detailed in Figure E1.

Immunostainings and tissue characterization

Fluorescent proteolipid protein (PLP) immunoreactivity staining was performed as previously published.[17] Briefly, frozen tissue slides were dried at room temperature, fixed with 4% PFA at room temperature for 10 min, washed with PBS and incubated with blocking solution (10% normal species serum (NSS) in 0.05% Tween20 (Sigma-Aldrich)) for 30 min. Primary antibodies for PLP (1:300; Serotec, MCA839G), UEA-I (1:1000, Vector Labs B-1065), HLA-DR (1:500; Hybridoma) and Fibrinogen-FITC (1:300, Dako, F0111) was diluted in 1% NSS in 0.05% Tween20-PBS and applied on the slides overnight at 4 °C. Tissue sections were washed in PBS and incubated for 1 h with Alexa fluorophore-conjugated secondary antibody. Nuclear staining was performed by using Hoechst fluorescent DNA stain (33258, Thermo Fisher Scientific) for 1 min. Images were acquired as a 20x overview scan at the Olympus VS200 slide scanner. Following, visual characterization was performed using QuPath software (version 0.4.4) to aid classification based on full (normal), diffuse (dirty appearing white matter) and absent (lesion) PLP immunoreactivity.[22]

Regions of interest selection and data extraction

In a pilot study, tissue samples from three non-neurological control (NNC) and five people with multiple sclerosis (PwMS) were analyzed using one thin tissue section for each subject. A multimodal approach was used to select ROIs unambiguously. In particular, a region had to fulfil two criteria to be considered as an ROI: i) have a morphologically well-defined border from surrounding areas based on PLP and HLA-DR staining, ii) have a well-defined chemical profile based on ion images acquired using PA nano-DESI MSI. Morphologically distinct areas

were defined according to the section “Immunostainings and tissue characterization” and stratified into normal, diffuse and absent PLP categories. (Fig E7). Chemically distinct areas were defined by segmenting the PA nano-DESI MSI ion images based on the spatial distribution of monoacylglycerol, phosphatidylcholine, phosphatidylethanolamine, prostaglandin, free fatty acid and sterol lipid classes. In total, 55 ROIs were identified: 19 normal PLP, 20 diffuse PLP, and 16 absent PLP (Table S8).

To compare the abundance of cholesterol and oxidized cholesterol products, average pixel intensities were extracted from each ROI using the i2i software[11] and the fractional abundance (FA) was calculated (Eq 3). In Eq. 3, the FA is calculated for each oxysterol based on its intensity over the sum of intensities of cholesterol (ST1) and all selected oxysterols (ST3, ST4, ST7).

$$FA = \frac{Int_{oxysterol}}{\sum(Int_{oxysterols} + Int_{cholesterol})} \qquad \textit{Eq. 3}$$

Subsequently the cholesterol oxidation level was defined according to Eq 4, where FA is the fractional abundance of the molecule and ON is defined by the additional oxygens in the sum-composition formula compared to cholesterol (0 for ST1, 1 for ST2, 2 for ST3 and ST4, and 4 for ST7).

$$Oxidation\ level = \sum(FA_i \times ON_i) \qquad \textit{Eq. 4}$$

Statistical analysis

Statistical analysis was performed and boxplots were created with R v4.3.2 in RStudio.[23] The normality of the data was tested using the Shapiro-Wilk test. The pilot study cohort was balanced for age and post mortem delay, and covariate significance of the distinct patient was tested using a Kruskal-Wallis test. Since it was significant in some cases and not significant in others, we decided to perform non-parametric one-way testing but denote each donor by color codes as per Table S8. In multi-group comparisons, Kruskal-Wallis test was performed, followed by Dunn´s post-hoc test where significant difference was indicated. For the two-group comparisons, Wilcoxon test was used. FDR correction was performed in all analyses with the Benjamini-Hochberg method. Significant differences are indicated in the plots as follows. *:p<0.05, **:p<0.01, ***:p<0.001, ****:p<0.0001. The results of statistical tests are detailed in Table S10. In the boxplots, black line denotes the median, the white dot the mean, the top and bottom edges of the box the interquartile range, and the whiskers extend to the minimum and maximum datapoints.

Hierarchical clustering-based heatmaps and Pearson correlation heatmap and PLS-DA plots were created using MetaboAnalyst 6.0.[24] Missing data points were imputed by LOD (1/5 of the lowest intensity). For hierarchical clustering, values were Z-scored, Euclidian distance measure was set, and autoscaling was set based on samples.

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Data availability

Data has been uploaded to figshare

Code availability

The source code and the compiled version of the software will be made available upon acceptance of the manuscript at https://github.com/LanekoffLab

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Author contributions

I.L. and J.L. conceived the study in its initial form. J.L. and R.M. wrote the code for the SSN. V.V.S., J.L., and G.T. performed mass spectrometry imaging experiments, analyzed and visualized the data. C.H. performed immunostaining experiments. G.T. and C.H. performed tissue classification. G.K. provided samples and critical feedback. I.L., V.V.S., and G.T. wrote the original manuscript. All authors contributed to reviewing and correcting the manuscript and gave consent to the final version.

Competing interest

The authors declare no competing interests.

Extended data figures

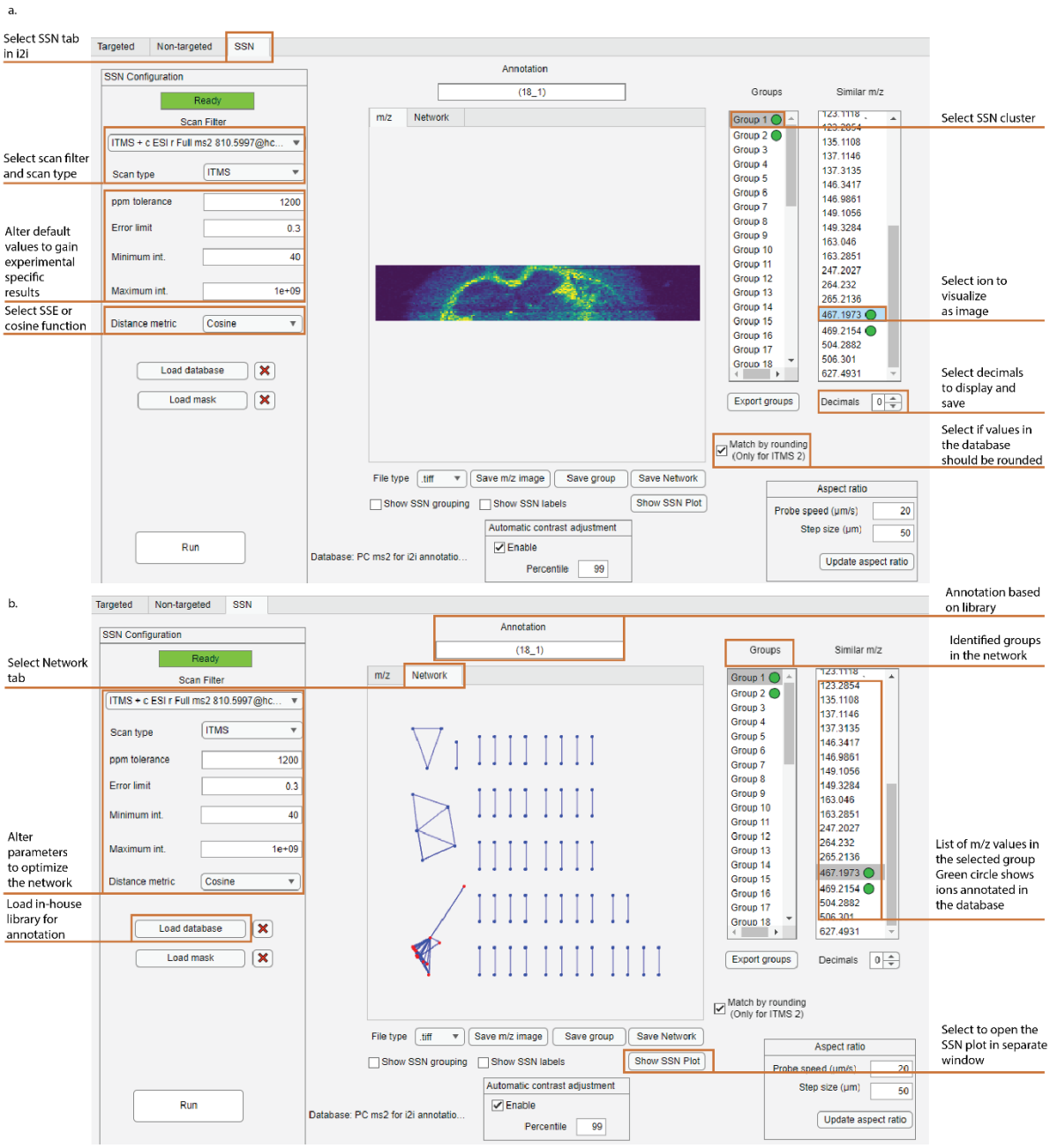

Figure E1. GUI of the new SSN tab in i2i. The operator selects the scan filter and scan type based on the loaded data together with the parameters. Default parameters are suggested although optimization may be required for each scan filter depending on the complexity and intensity of the detected ions **(a)**. The SSN automatically clusters ions in the selected scan (here, FTMS, $FTMS^2$ or $ITMS^2$) into networks based on either the sum of squared error (SSE) or the cosine spatial similarity metric of the ion distributions. The network group and *m/z* of the ion image to be displayed is selected together with the relevant number of decimals or rounding, depending on the mass resolution. The product ion image in the *m/z* tab shows the acyl chain 18:0 (*m/z* 467.2 of $[PC(36:1)+Na]^+$ (*m/z* 810.2) with the annotated ions marked with a green dot. In the network tab, the resulting SSN is displayed **(b)**. The network shows nodes of *m/z* values that are clustered based on their spatial distributions. The number of the individual SSN groups are ordered by size, where Group 1 is the largest group. The graph layout, including the length of the lines within each SSN group is automatically set by MATLAB and has no value for the interpretation of the SSN. To interrogate the SSN, the user can select groups and their *m/z* values in the list box next to the displayed SSN. Alternatively, by opening the SSN as a new window, the user can hover over the displayed SSN to view information on each node in real time and save the plot. For automatic annotation of the *m/z* values in the SSN, based on ppm difference or rounding, the user can upload a database containing *m/z* values and known names of the corresponding ions for matching (Tables S-S4, S7). For experimental details see Dataset 1 in Table S9.

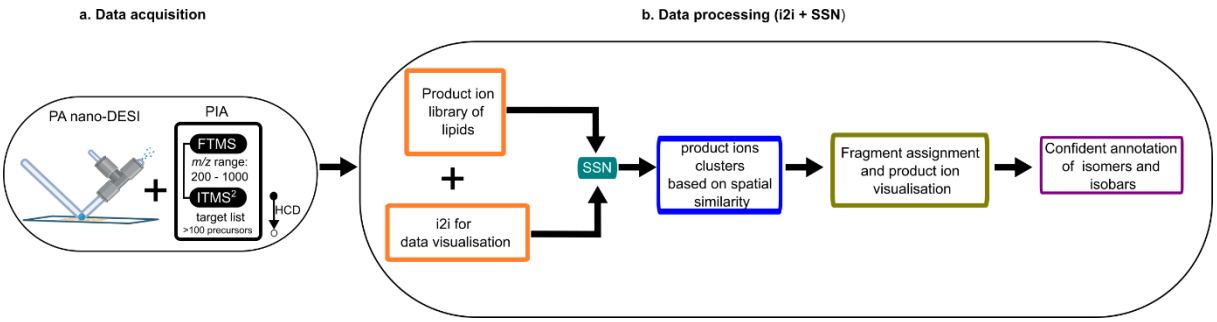

Figure E2. Summary of PIA SSN workflow for imaging and metabolite annotation with PA nano-DESI. Tissue is placed on the PA nano-DESI platform and molecules are desorbed into the nano-DESI solvent liquid bridge between the two fused silica capillaries (50/150 µm i.d./o.d.). Ions generated from endogenous molecules through electrospray ionization are used to first fill the orbitrap for a high-resolution FTMS full scan, taking 1024 ms. Until the next FTMS scan event, the ion trap is filled for targeted $ITMS^2$ scan events after HCD. Depending on the ion abundance, and hence the ion accumulation time, the number of $ITMS^2$ scans during the FTMS scan can vary. The complexity of the product ion scan will depend on the size of the precursor isolation window and the present isobars and isomers at the targeted *m/z* value. The SSN is performed for all pixels in the ion image and based on the selected scan event, which can be performed in MS^1 or MS^2 and with any mass analyzer. The SSN outputs a graphical view where each cluster of ions has a similar spatial distribution, and therefore stem from the same precursor ion. The product ions can automatically be annotated using an uploaded *m/z* database and ion images from each cluster are readily interrogated in the "m/z" window to ensure the distribution similarity. The ion distribution therefore works as a third orthogonal parameter to high mass resolution data and product ion spectra to ensure high confidence in annotation of ions, including isomers and isobars.

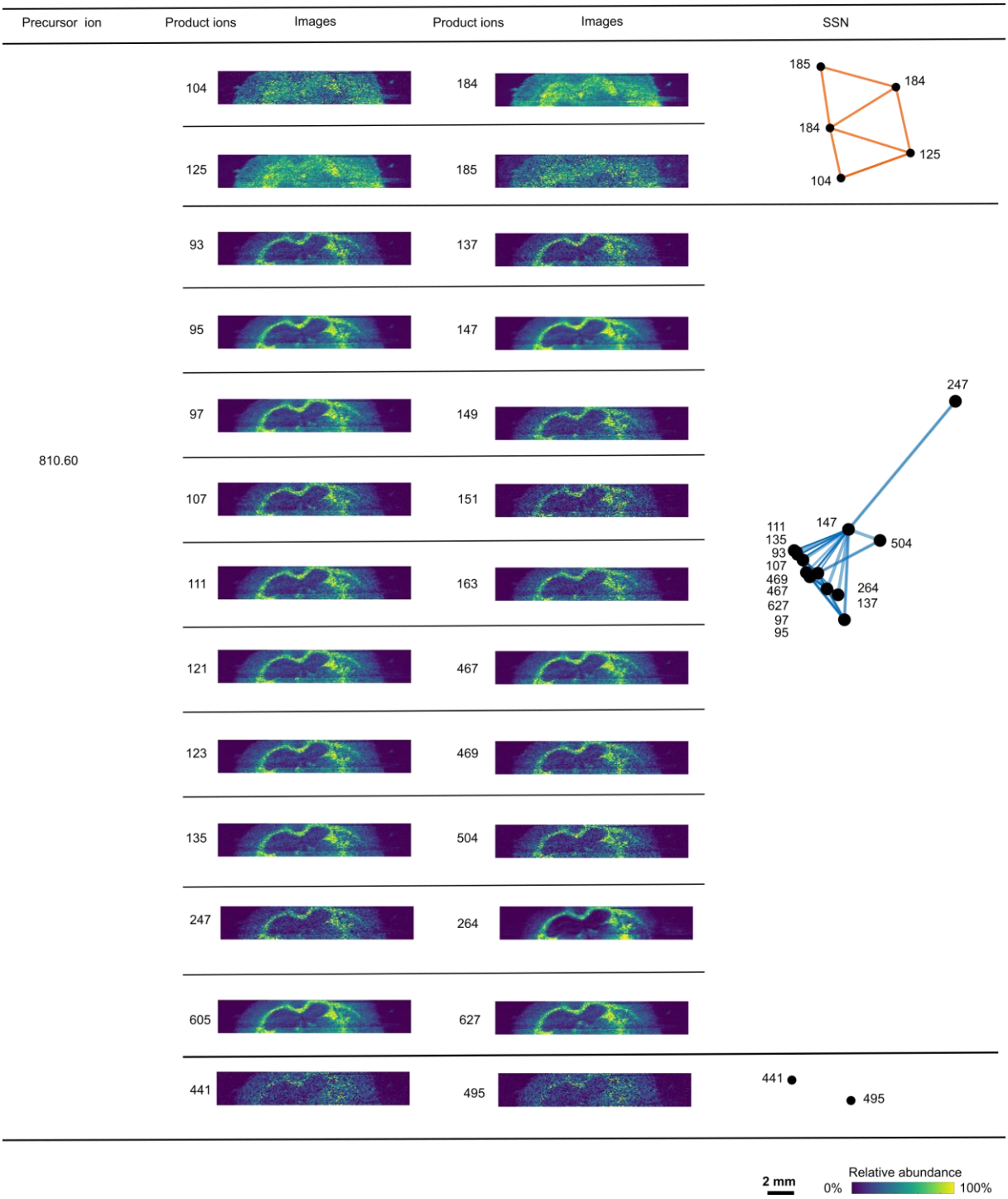

Figure E3. Product ion images networks from PIA SSN. The *m/z* values and corresponding product ion images acquired from the precursor window of 810.60 ± 0.35 Da using PIA of a mouse brain tissue section. The subsequent SSN reveals that product ions are separated into two groups based on their spatial distribution, as shown by the orange and blue SSN, suggesting the presence of two structurally different precursors. Two additional disconnected nodes are present, indicating a third, but less abundant precursor. From top to bottom the represented ions are [PC 38:4+H]$^+$, [PC 18:0_18:1+Na]$^+$, and [PC 16:0_20:1+Na]$^+$. For experimental details see Dataset 1 in Table S9.

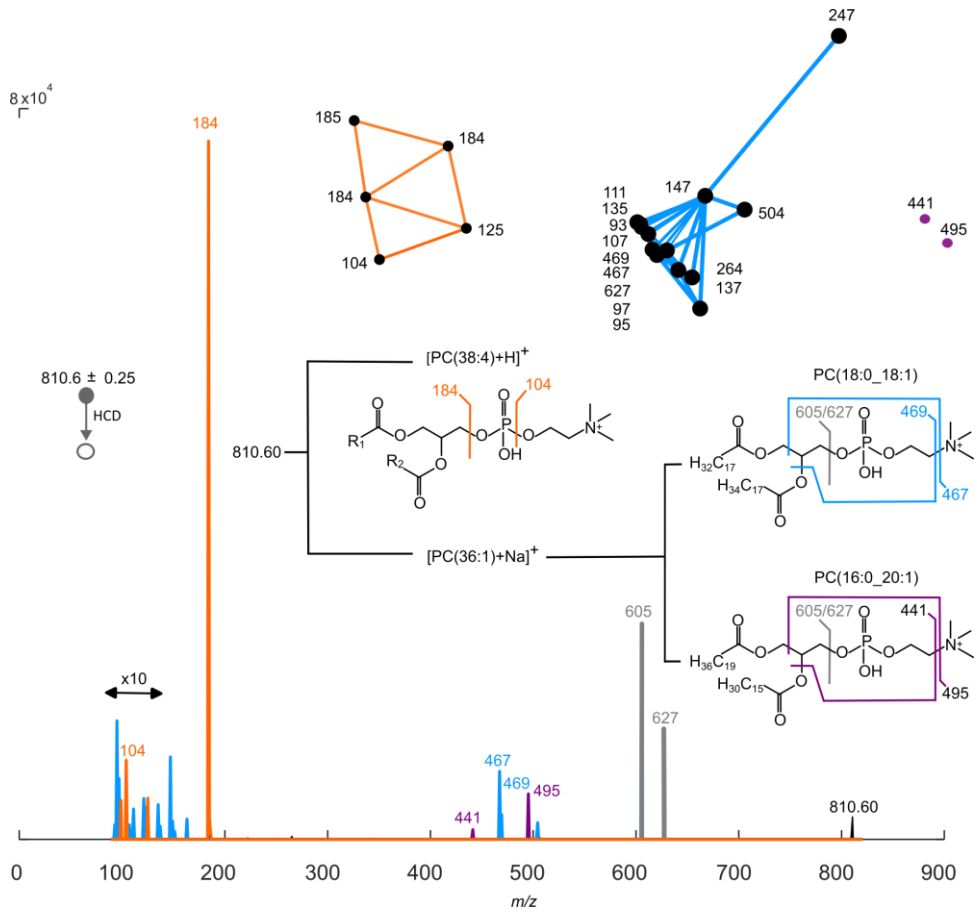

Figure E4. **SSN deconvolutes product ion mass spectra and facilitates annotation of isobars and isomers.** The convoluted ITMS^2 mass spectrum produced from the product ions of all precursors in the 810.6 ± 0.35 window is readily deconvoluted by SSN. The product ions corresponding to the blue and orange SSN are shown in blue and orange, respectively. This shows that the two distinct isobars of $[PC(38:4)+H]^+$ and $[PC(36:1)+Na]^+$, which have different spatial distributions, both originate from the precursor ion at *m/z* 810.60. Furthermore, the data reveals that $[PC(36:1)+Na]^+$ consists of the two isomers PC(18:0_18:1) and PC(16:0_20:1). For experimental details see Dataset 1 in Table S9.

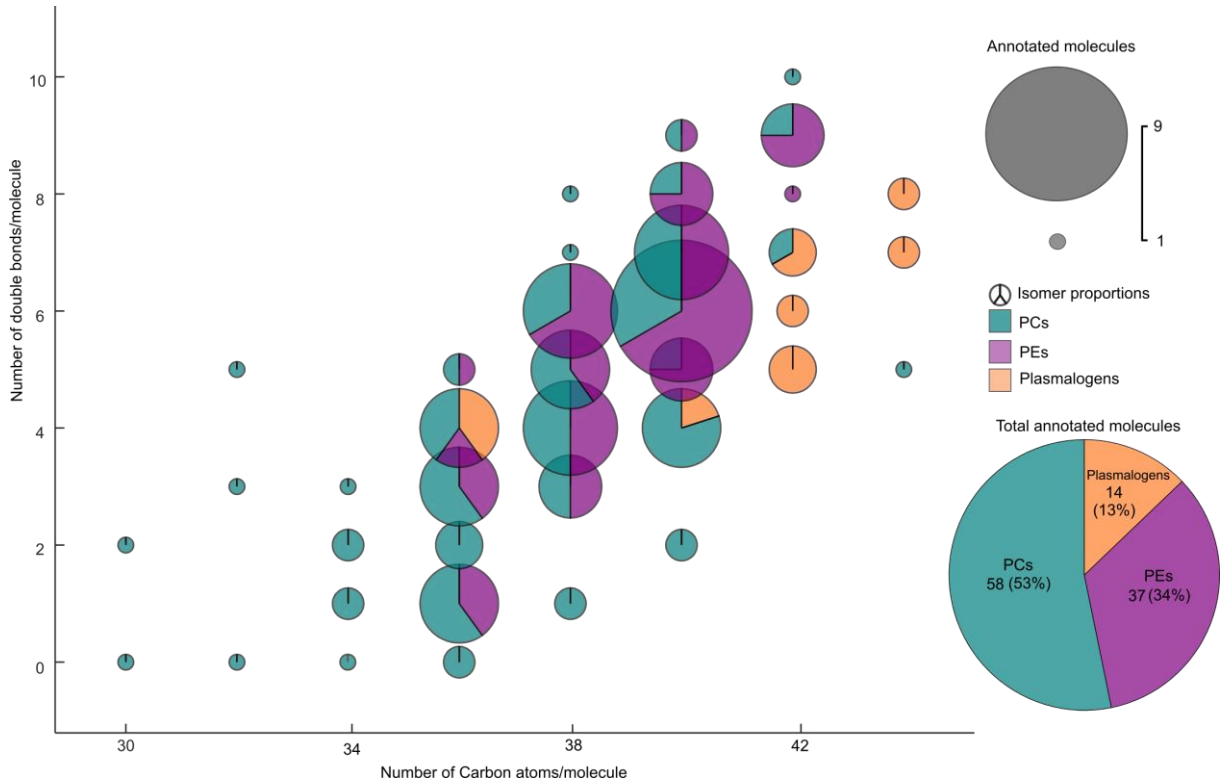

Figure E5. **PIA SSN uncovers hidden isomers and isobars.** Total number of imaged and annotated PC, PE and PE-plasmalogen lipids in positive ion mode using PIA SSN on a mouse brain cross-sectional tissue section with 108 fragmentation windows of ± 0.35Da. 34 of the 108 fragmentation windows were dedicated to phospholipids as sodiated adducts. In these mass channels we annotated in total 134 phospholipid adducts with isobaric and isomeric precision. Out of these, 109 were unique phospholipid structures corresponding to 58 PCs, 37 PEs, and 14 PE-plasmalogens. The color of the bubbles represents the respective lipid classes and the size corresponds to the number of annotated species. For experimental details see Dataset 1 in Table S9.

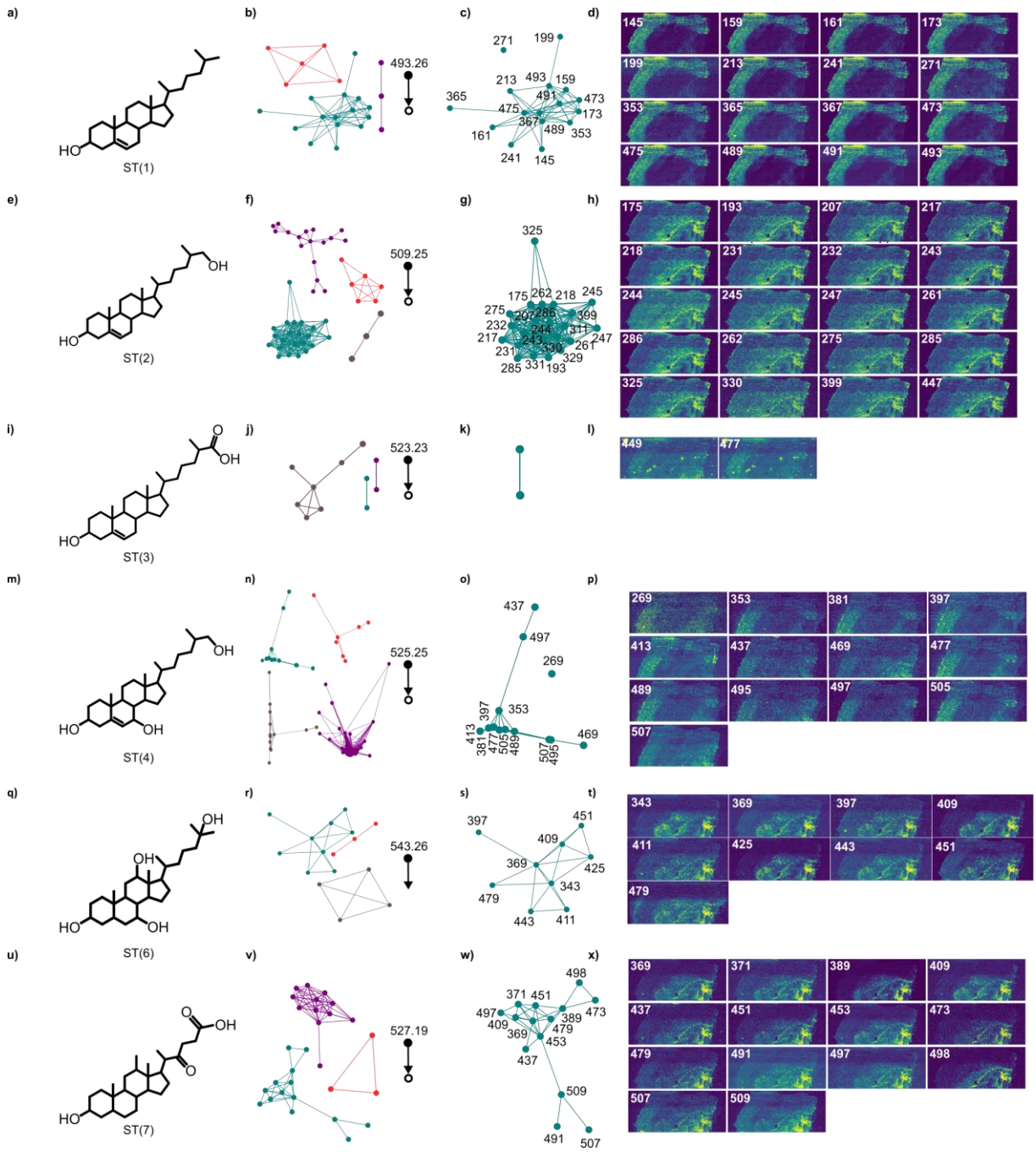

Figure E6. The SSN deconvolutes complex ITMS^2 spectra produced by fragmentation of co-isolated precursor ions. Characterization of oxysterol structures detected by PA nano-DESI MS^{2}I of human multiple sclerosis brain tissue. Structural annotation of cholesterol and oxidized cholesterol products using HCD of $[M+^{107}Ag]^+$ adduct in ITMS^2. **a-d** cholesterol, **e-h** hydroxycholesterol **i-l** hydroxycholestenoic acid, **m-p** dihydroxycholesterol, **q-t** cholestantetraol, **u-x** homodeoxycholic acid. For each targeted molecule, SSN and all product ions in the SSN are provided. One additional structural annotation (dihydroxy-cholestenoic acid) is shown in figure 2. For experimental details see Dataset 5 in Table S9.

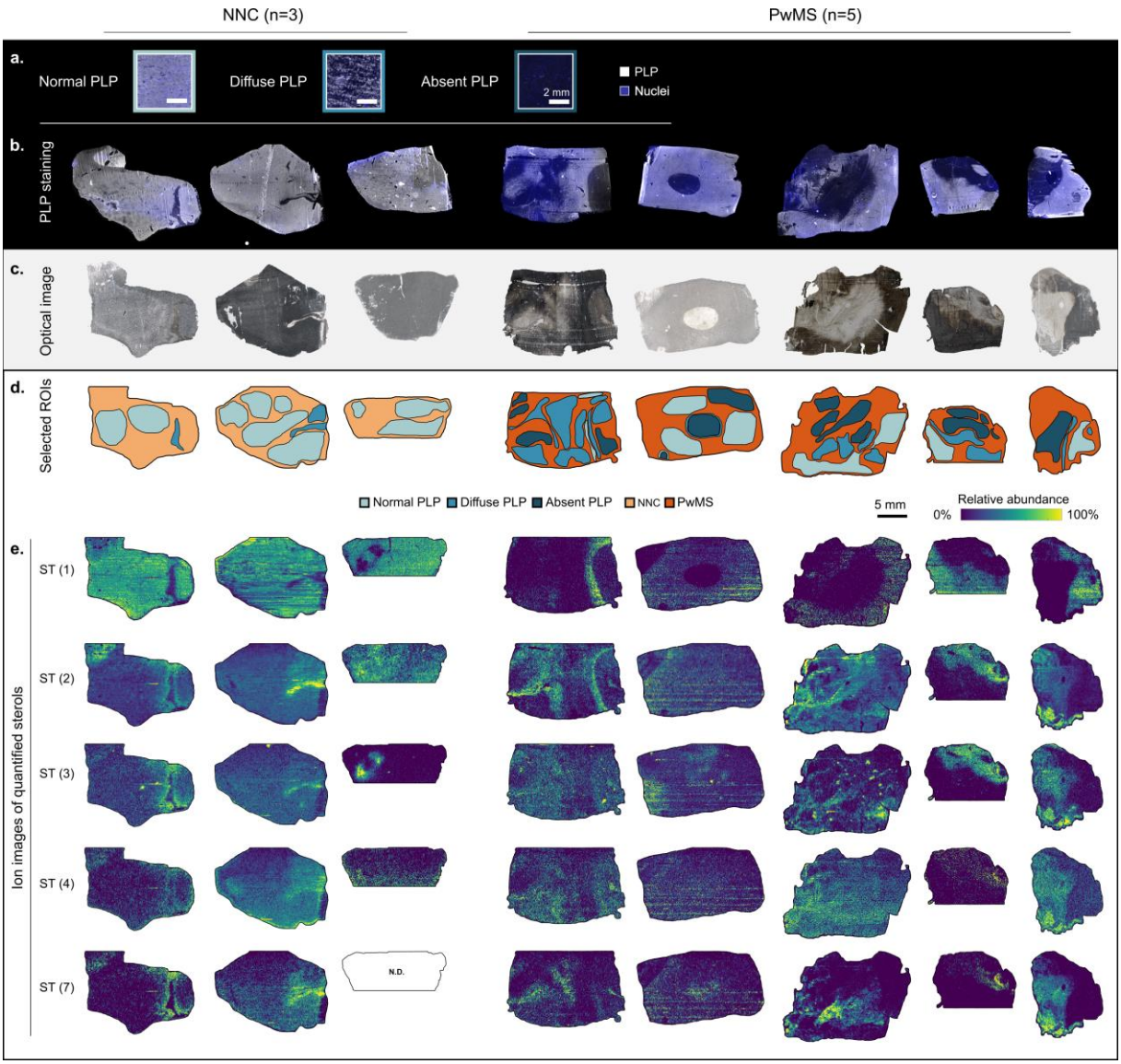

Figure E7. Multimodal imaging of cholesterol and oxidized cholesterol products in human white matter brain tissue sections. Representative classifier images of fluorescent proteolipid protein (PLP) immunoreactivity were acquired as stated in the Methods section. Scalebar is 2 mm. **(a)** Full tissue scans of PLP immunoreactivity of human brain tissues (NNC=3, PwMS=5). Image intensities are scaled individually. **(b)** Brightfield microscopy images acquired using a Meyer Instruments PathScan Enabler IV slide scanner device. **(c)** Resulting ROIs for each tissue section, colored according to the PLP classification (light blue: normal PLP, medium blue: diffuse PLP, dark blue: absent PLP). **(d)** Ion images of cholesterol and oxidized cholesterol products in human white matter brain tissues from NNC and PwMS normalized to TIC and the 99th percentile intensity. Scalebar is 5 mm and applies to images **b-e**. N.D.: not detected. Subsequent tissue sections are used for PLP and MSI imaging modalities.

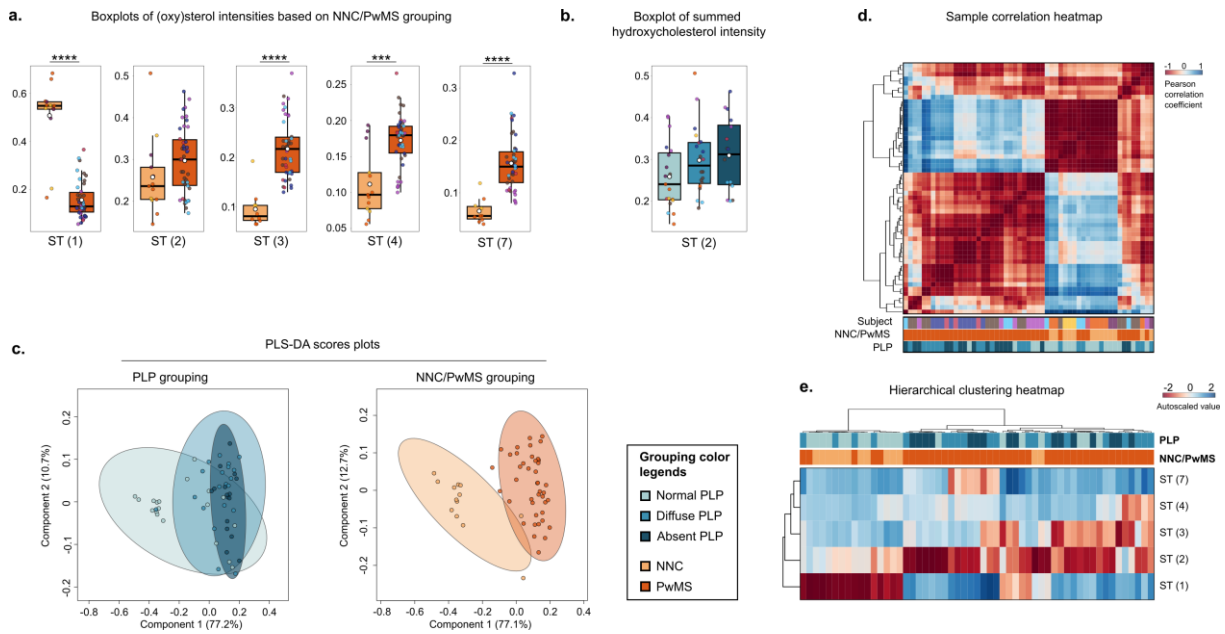

Figure E8. Differentiation and clustering of brain tissue regions based on their oxysterol profiles. (a) Boxplots representing the fractional abundance of detected sterols between ROIs corresponding to NNC (light blue) and PwMS (orange) subject samples. Note that since quantitation is based on MS^1, the two detected isomers 24-hydroxycholesterol and 27-hydroxycholesterol are denoted together as ST(2). All other mass channels are confirmed by $FTMS^2I$ to only contain the annotated sterol. Significant differences were observed for all the molecules, except ST(2). To define significance, Wilcoxon test with Benjamini-Hochberg FDR correction was performed (*:p<0.05, **:p<0.01, ***:p<0.001, ****:p<0.0001). Coloring of jitters display data from individual subjects as denoted by Table S8. **(b)** Boxplot representing the fractional abundance of ST(2) showing non-significant differences between ROIs corresponding to normal PLP (light blue), diffuse PLP (medium blue), and absent PLP (dark blue) subject samples. For additional boxplots, see Figure 2 in the main text. **(c)** PLS-DA analysis of sample grouping according to PLP and donor type (NNC vs PwMS) based on oxysterol profiling of ROIs. We observed only a slight differentiation of normal PLP ROIs from diffuse and absent PLP ROIs. However, a considerable differentiation is seen when the grouping is based on the subjects (NNC vs PwMS). This observation is further strengthened by the Pearson correlation heatmap of ROIs based on their (oxy)sterol profile **(d)** and the hierarchical clustering heatmap of (oxy)sterols across all ROIs **(e)**. For hierarchical clustering, Ward method with Euclidian distance measures was used and values were autoscaled within each ROI sample. The coloring of hues for the Pearson correlation coefficients and the autoscaled values for hierarchical clustering are displayed in the top right corner. We observed excellent clustering based on NNC/PwMS subject grouping. The dominant sterol in NNC samples was cholesterol (ST(1)), while for the PwMS samples it was hydroxycholesterol (ST(2)). The latter (although not statistically different) shows an increased abundance in PwMS. The differentiation is predominantly based on patient type, and slightly based on the PLP abundance of each ROI. However, interpatient differences are considerably larger compared to the differences due to classification parameters, therefore a follow-up study with larger sample size is required to deduct further biological conclusion.