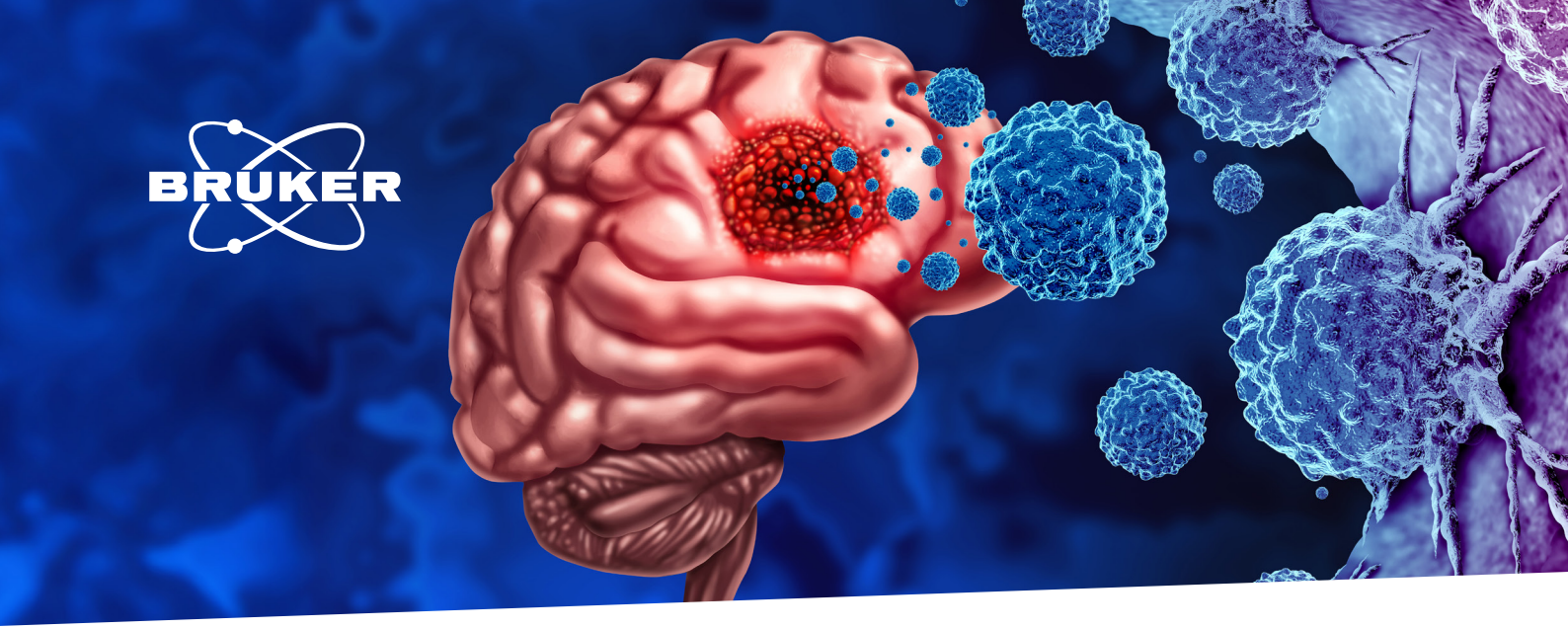

Spatial and cell-type specific lipid analysis for in situ assessment of intratumor heterogeneity

Understanding glioma by combining SpatialOMx® and MALDI HiPLEX-IHC workflows.

Abstract

Glioblastomas as astrocytic tumors are characterized by strong intratumor heterogeneity and have very poor prognosis. Understanding glioma pathology requires examining cellular features beyond just genomics and proteomics. MALDI Imaging allows researchers to probe these features within a spatial context, meaning they can see where specific molecules are located in tissue. Here we demonstrate for the first time that the powerful combination of MALDI HiPLEX-IHC and SpatialOMx® enables the integrated, spatially resolved lipid and protein analysis of tissue specimens, including solid tumors. These results show unique lipid features in combination with cellular and tissue type markers, provide essential knowledge and help to uncover the underlying biology of glioblastomas.

Keywords:
MALDI HiPLEX-IHC, SpatialOMx®, Glioblastoma, Astrocytoma, Cancer, Lipidomics, MALDI Imaging, SCiLS™ Lab, SCiLS Ion Image Mapper, Multiomics

Introduction

The integration of LC-based analysis with spatial proteomics and lipidomics enables simultaneous visualization of different classes of molecules, the mapping of lipids, and protein expression on the same tissue section, producing multiple levels of information. The innovative combination of two workflows, SpatialOMx with MALDI HiPLEX-IHC (using technology from AmberGen Inc.*), enables elucidation of the complex biochemical information found in clusters of cells in the tumor microenvironment.

SpatialOMx leverages both classical LC-MS/MS PASEF® workflows with MALDI Imaging, which allows researchers to gain a more comprehensive understanding of the biomolecules expressed in cancer tissues and their relationship to tissue pathology. 4D-omics data provides the highest confidence in lipid identification, and MALDI Imaging allows for locating specific biomolecules within a tissue section. Utilizing laser-based post-ionization (MALDI-2) within the MALDI Imaging experiment leads to better sensitivity, resulting in an enhanced number of detectable lipid classes typically opaque to traditional MALDI. Finally, the MALDI HiPLEX-IHC workflow is a cutting-edge targeted spatial proteomics technique that seamlessly combines two methods: MALDI Imaging and immunohistochemistry. This novel approach enables the visualization and analysis of the distribution of multiple intact proteins with high plexing capabilities [1].

* Miralys™ photocleavable mass tag imaging probes plus their high-plex and multimodal oriented workflows are patented by AmberGen Inc. To learn more about Miralys imaging go to AmberGen.com. To purchase probes contact Info@AmberGen.com.

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The interplay between lipids and proteins gives a comprehensive multiomics view that allows a detailed understanding of the tumor heterogeneity and microenvironment. The integration of both data sets in SCiLS™ Lab combines cellular subtyping with biochemical information.

A compelling application of this approach is to study gliomas, including glioblastomas (GBM), the most malignant form of glioma. The aggressive behavior of GBM, along with their significant resistance to adjuvant therapy, contributes to patient 5-year survival rates below 10%. GBMs are characterized by enormous heterogeneity within tumor cells and the immunosuppressive tumor microenvironment, partially caused by dysregulated lipid metabolism. Combined, these traits influence chemotherapy resistance and local cancer cell dissemination [2]. While intratumor heterogeneity and the tumor microenvironment have been studied, the spatial context is not well understood but could provide important insights into the pathophysiology. Specific lipid species are known to be present in cancer tissue, but their role in the spatial dimension is unknown.

These workflows not only enhance our understanding of gliomas but also underscore the importance of the cell and tissue type specific lipidome [3].

Methods

Lipid Profiling Using LC-MS/MS PASEF

All samples were anonymized and obtained with informed consent, following ethical guidelines for human research. Bulk tumor tissue pieces from three glioblastoma patients (WHO grade IV) and a less malignant astrocytoma (WHO grade III) patient were homogenized. Lipids were extracted via MTBE:water (H_2O) 750 µL:190 µL phase separation and used for downstream 4D-Lipidomics™ (retention time, *m/z*, MS/MS fingerprints, intrinsic CCS values). Data were collected on a timsTOF fleX with a standard LC-MS/MS PASEF Lipidomics setup generating a Target List in MetaboScape® for annotation of the imaging features in SCiLS Lab.

MALDI Imaging of Lipids

Fresh frozen tissue from the same patients were cut into 10 µm tissue sections and mounted on IntelliSlides®, coated with poly-*L*-Lysine (Bruker Daltonics GmbH & Co. KG, Bremen, Germany). Slides were coated with DHAP matrix using a M3+sprayer (HTX Technologies, Chapel Hill, USA) and measured on a timsTOF fleX MALDI-2 (20 µm spatial resolution, positive ion mode, TIMS enabled, MALDI-2 enabled) from *m/z* 200-1400. The TIMS parameters were set with a mobility range of $1/K_0$ 0.63-1.87 (V*s/cm²) and 200 ms ramp time.

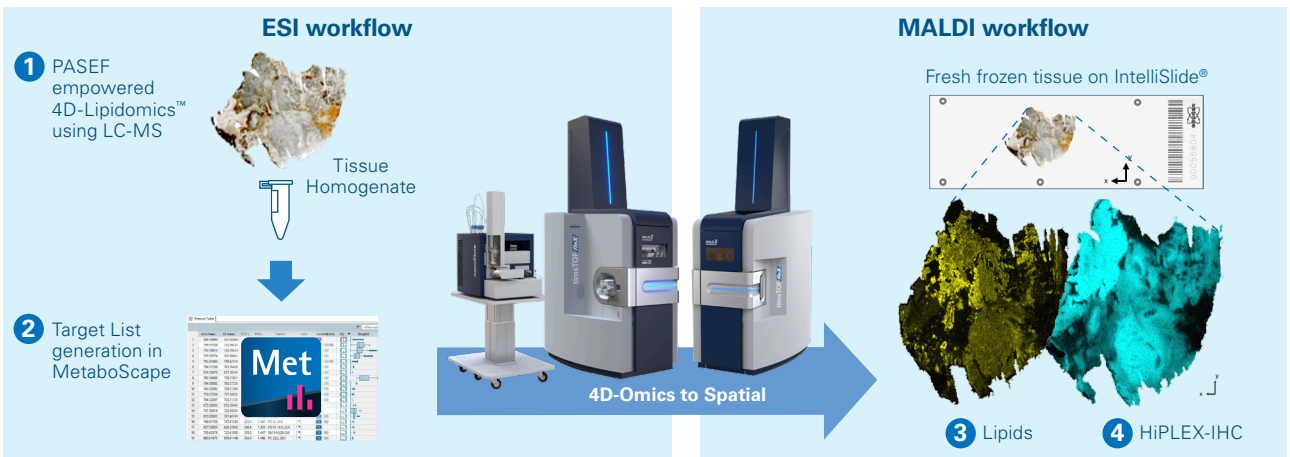


Figure 1. Overview of the workflow.
The first step included a standard LC-MS/MS PASEF Lipidomics analysis of homogenized bulk tumor tissue using the ESI side of the timsTOF fleX (1), which resulted in a Target List in MetaboScape (2). Second, tissue slices of the samples were coated with DHAP matrix and a MALDI Imaging experiment in the lipid mass range was performed using the MALDI side of the same timsTOF fleX instrument (3). After that, the matrix was washed off and the PC-MT antibodies were applied for the HiPLEX-IHC measurement (4). The Target List from the 4D-Lipidomics experiment was used for identification of lipid species in the MALDI Imaging experiment. All images can be colocalized and overlayed using SCiLS Ion Image Mapper.

MALDI HiPLEX-IHC

After the lipid MALDI Imaging experiment, the matrix was removed and the same tissue samples were subjected to MALDI HiPLEX-IHC staining according to a previously published protocol [1]. A total of 16 photocleavable mass-tagged antibodies (PC-MT), available from AmberGen Inc. (Billerica, MA), (*CD20*, *PDGRF-β*, *PTEN*, *CD68*, *VIM*, *Collagen 1A1*, *CD45RO*, *PanCK*, *CD4*, *NeuN*, *Ki67*, *CD8α*, *GFAP*, *FoxP3*, *Na/K ATPase*, *Histone-H2A.x*) were used for the staining at a concentration of 2.5 µg/mL, except FoxP3 at 10 µg/mL. Slides were coated with HCCA matrix using an M3+sprayer followed by recrystallization (1 mL 5% IPA in an oven at 55°C, 1 min). Images were acquired with the timsTOF fleX MALDI-2 (20 µm spatial resolution, positive ion mode) from *m/z* 900-1600.

Data analysis

Data visualization was done using SCiLS Lab 2024b software (Bruker Daltonics GmbH & Co. KG, Germany). Lipid annotations were assigned with MetaboScape 2024b (Bruker Daltonics GmbH & Co. KG) using the targeted lipid list generated from the LC-MS/MS results of tissue homogenate. SCiLS Ion Image Mapper was used to transfer the spatial coordinates of the detected antibody mass-tags into the lipid image SCiLS Lab files.

Results

Lipid Imaging

The 4D-Lipidomics analysis of homogenized bulk tumor tissue yielded a comprehensive Target List that includes numerous individual lipid species across several lipid classes. Matching this Target List to the MALDI Imaging data resulted in 94 hits. A statistical evaluation of these lipids in SCiLS Lab, using techniques such as ROC analysis, revealed novel insights into the spatial biology of gliomas. A comparison of three glioblastoma and one astrocytoma (as a control) samples revealed specific lipid classes to be differentially abundant. Figure 2 shows that specific phosphatidylcholines (PCs), e.g. PC 36:1, are strongly overexpressed in glioblastomas. PCs are

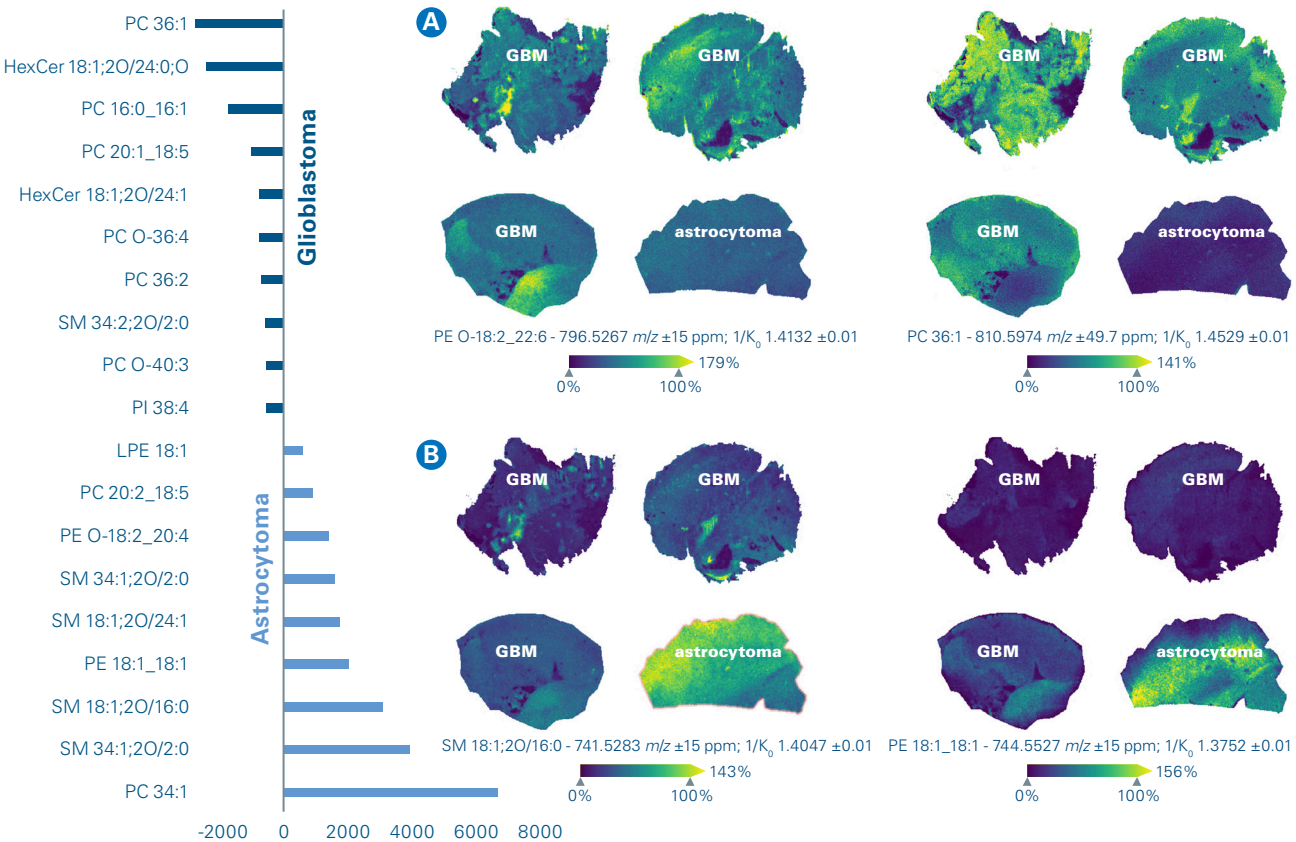
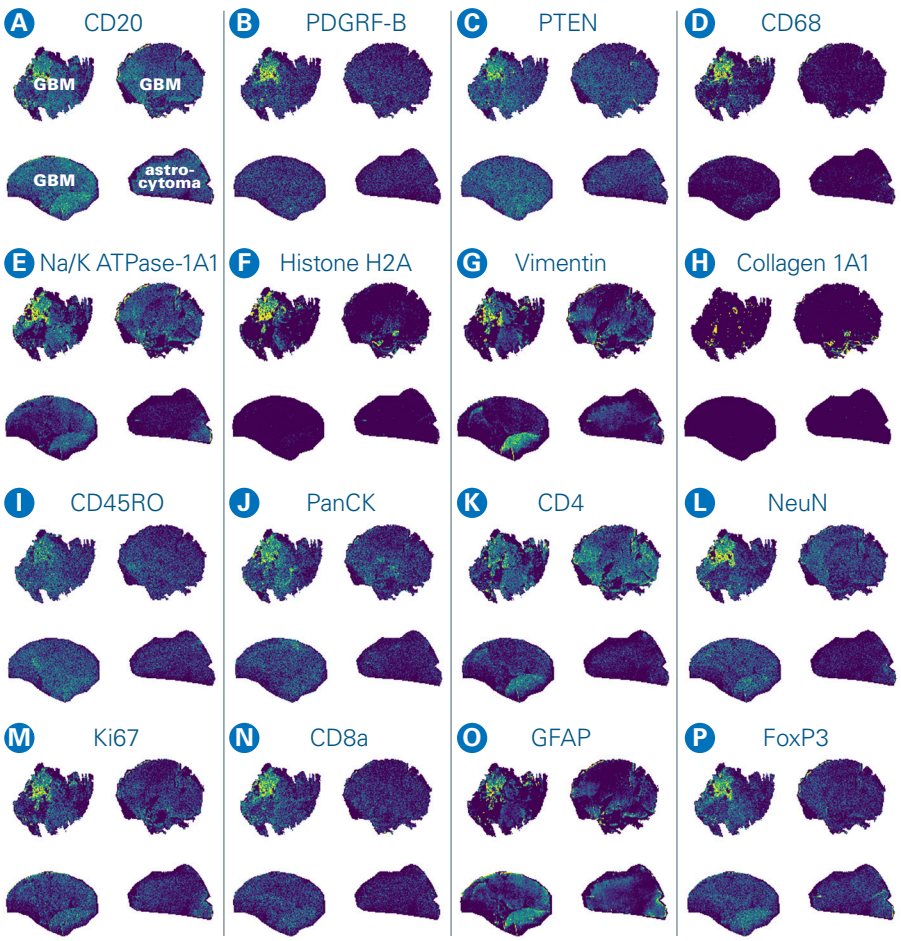


Figure 2. Examples for identified lipids upregulated in glioblastoma (dark blue) and in astrocytoma (light blue).
Respective ion images reveal the overexpression for PE-O 18:2_22:6 and PC 36:1 in GBM (A) and for SM 18:1;2O/16:0 and PE 18:1_18:1 in astrocytoma (B).

a major component of the cell membrane, and it has been shown that this lipid class is highly expressed in GBM. Another representative lipid for the group of upregulated species in GBMs is found to be PE-O (18:2_22:6), shown in Figure 2A. Some lipid classes, such as sphingomyelins (SM) and (lyso)phosphatidylethanolamines ((L)PE), are lower abundant in glioblastomas as shown by two examples in Figure 2B, namely SM (18:1;2O/16:0) and PE (18:1_18:1).

MALDI HiPLEX-IHC

Following lipid imaging, the same tissues were stained with 16 PC-MTs purchased from AmberGen, to target and image proteins of interest. Selection of the antibodies included some that are indicative of tumors (PanCK, GFAP, VIM, PTEN, FoxP3, Na/K ATPase, Histone-H2A.x), development and progression (Ki67, NeuN, PDGFR -β), immune cells in the tumor microenvironment (CD68, CD8α, CD4, CD20, CD45RO), as well as a general structural marker (collagen 1A1). MALDI HiPLEX-IHC Imaging on cell type specific antibodies revealed different cellular compositions in the four investigated brain samples. Glia cells were more evenly distributed over the tissue and showed variation between the four samples compared to immune cells. CD4 T-cells appeared more intensely in the tumor tissue areas, while macrophages, visualized by CD68 marker, partially colocalized with collagen 1A1 in round structures, possibly corresponding to areas of stem cells. The spatial location of the tumor specific marker GFAP fits well to the tumor area confirmed by the pathologist annotation in the H&E stain (data not shown). Macrophages marked with CD68 are attracted to and migrate towards the tumor. The distribution of other used antibodies fits the samples as expected, e.g. NeuN as a neuronal protein is evenly distributed in the tumor at slightly lower abundance than outside the tumor. Vimentin is associated with tumor area, which is a filament associated with astrocytes and should be increased during tumor progression. All ion images of the mentioned markers can be found in Figure 3.



	Protein	*m/z* of PC-MT
A	CD20	997.53
B	PDGRF-B	1125.63
C	PTEN	1132.6
D	CD68	1216.75
E	Na/K ATPase-1A1	1222.79
F	Histone H2A	1226.82
G	Vimentin	1230.84
H	Collagen-1A1	1234.87
I	CD45RO	1251.68
J	PanCK	1288.72
K	CD4	1293.75
L	NeuN	1308.71
M	Ki67	1320.76
N	CD8a	1350.76
O	GFAP	1377.78
P	FoxP3	1494.82

Figure 3
Ion images of the different marker proteins in the four biopsy samples (three glioblastoma, one astrocytoma as control) represented by their mass-tag (a-p) using the MALDI HiPLEX-IHC technology. Respective m/z values of the mass-tags are given in the table right.

Correlation of Lipid and Protein information

The SCiLS Ion Image Mapper transfers the ion images of the detected antibody mass-tags into the lipid image dataset maintaining the correct spatial relationship between sources and target measurement regions. This stand-alone tool enables spatial correlation analysis in SCiLS Lab by combining the lipid and MALDI HiPLEX-IHC data. Statistical tools identified colocalized features and provide new insight into the role of lipid metabolism in tumor progression, therapy response or immune cell defense. Figure 4A shows an overlay image of three markers (vimentin, collagen 1A1 and CD68) and three lipids (SM, PC and PE-O) which share the same localization in tissue. The specific areas highlighted by CD68, a marker for activated microglia and macrophages, and GFAP, a marker for astrocytes, underscore the intricate cellular composition within glioblastoma tissue. This correlation with lipids provides valuable insights into the metabolic and cellular interactions at play, demonstrating the power of lipid mapping in understanding tumor microenvironments (Figure 4B).

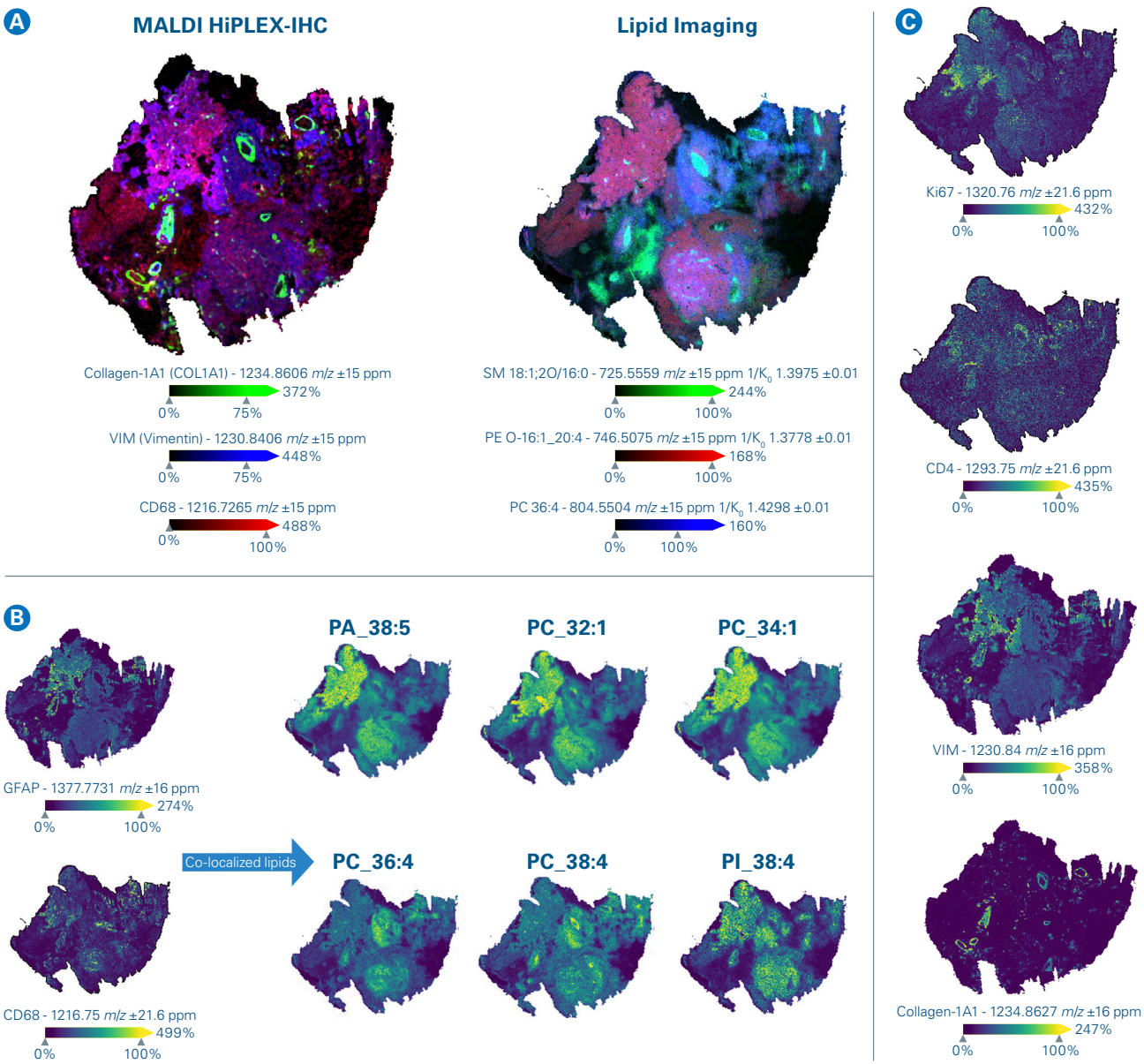


Figure 4.
Overlayed image of three MALDI HiPLEX-IHC markers in comparison to three lipid markers which showed the same distribution (A). MALDI HiPLEX-IHC data can be integrated in the same SCiLS Lab file as the MALDI Imaging lipid data to combine the data and co-localize different lipids to specific markers with statistical tools, as shown for GFAP and CD68 (B). Ion images of the photocleaved mass-tags for specific antibodies are shown for orientation in (C).

Conclusion

SpatialOMx and the MALDI HiPLEX-IHC workflow provide powerful tools for simultaneous visualization of biomolecules within tissue sections. The combination of untargeted lipidomics, spatial lipidomics, and spatial proteomics provide novel insights into the molecular composition of tumor tissues. This integration enables detailed exploration of pathophysiological processes and identification of disease associated biomolecules in complex tissues by merging high spatial resolution biochemical data with comprehensive molecular characterization and annotation, all within the context of detailed tissue morphology. Furthermore, these experimental modalities enable the study of the unique and patient-characteristic distribution of lipids and proteins in individual patient samples, facilitating a comprehensive multiomics approach to understand cancer biology in the era of personalized medicine.

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Learn more about MALDI HiPLEX-IHC and how to prepare samples successfully for targeted protein experiments in order to take your cancer biology research to the next level by viewing this webinar on demand.

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