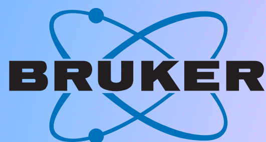

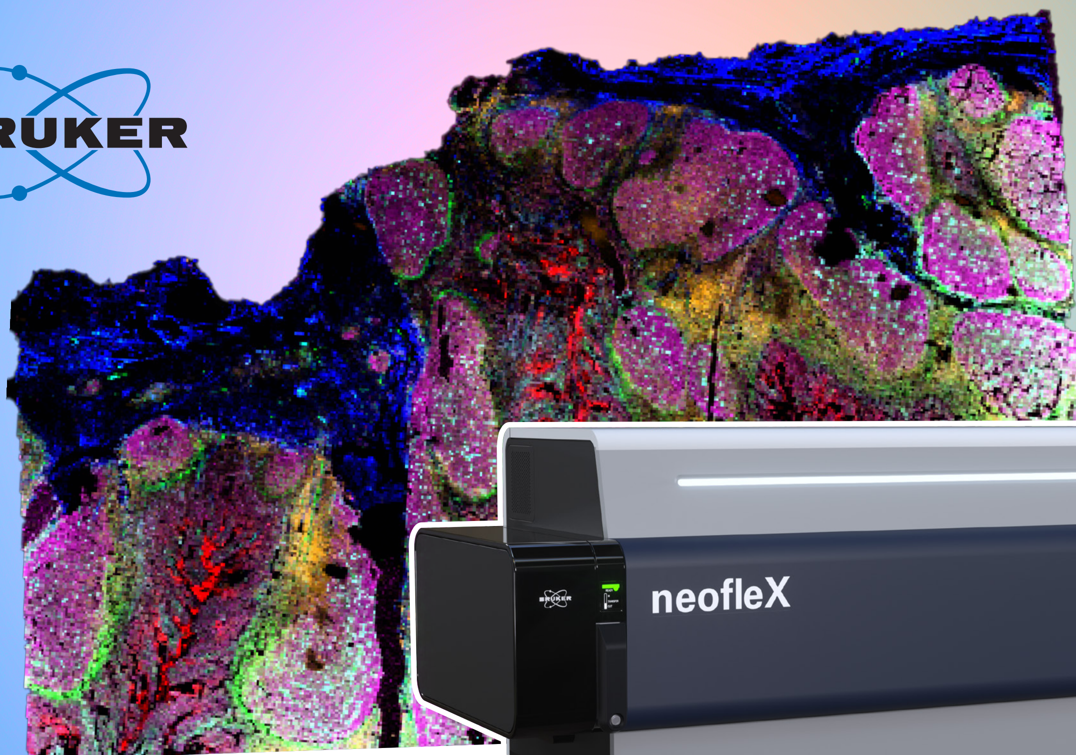

The neofleX Imaging Profiler: MALDI Imaging on a new benchtop axial MALDI-TOF mass spectrometer

Designed to expand your spatial biology utility to another dimension.

Abstract

The neofleX™ Imaging Profiler is a novel axial MALDI-TOF mass spectrometer with a benchtop footprint. It combines knowledge from three decades of MALDI hardware and software innovations in an inimitable powerhouse. With smartbeam 3D laser technology for true pixel imaging and enhanced imaging detectors, the instrument fulfills all requirements to augment your spatial biology research. Streamlined software workflows for ease-of-use combined with innovative self-diagnostics complement the robust technical configuration. The neofleX Imaging Profiler provides a toolkit to dig deeper into the molecular expression profiles of tissues and push your translational research to the next level.

Keywords:
MALDI Imaging, spatial biology, MALDI HiPLEX-IHC, SCiLS™ Scope, SCiLS™ Lab, neofleX

Introduction

Spatial biology is a rapidly evolving field aiming to understand biological processes on a molecular basis in the context of tissue architecture. The neofleX Imaging Profiler is ideally suited to visualize spatially targeted protein expression from tissue sections with a large field of view by MALDI HiPLEX-IHC. Moreover, it enables multimodal workflows to overlay spatial proteomics data with distributions of lipids, endogenous peptides, or enzymatically released glycans or peptides, and to match those profiles with tissue histomorphology. The robust design with latest technological innovations and software solutions makes it easy for new users to enter the field of MALDI Imaging.

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Integrated performance checks allow a self-diagnosis and ensure stable performance over large tissue cohorts. The system is capable of measuring MALDI Imaging mass spectra at a speed of 20 pixels per second, achieving a full field-of-view image with 20 μm pixel size from a typical tissue section of 1x1 cm^2 in less than 3.5 hours.

Here, the neofleX Imaging Profiler is characterized for different MALDI Imaging applications. Its excellent performance is demonstrated as a tool to complement spatial biology techniques, and as a novel player in the translational space.

Methods

Samples

Fresh-frozen sections from rat brain were cut on a cryo-microtome at 10 μm thickness and FFPE sections of a human colorectal cancer specimen were prepared at 5 μm thickness. All sections were mounted on IntelliSlides® (Bruker Daltonics GmbH & Co. KG, Bremen, Germany). A MALDI HiPLEX-IHC prestained mouse brain section using photo-cleavable mass tagged antibodies (Table 1) was purchased from AmberGen, Inc. (Billerica, MA, USA).

Table 1. Tagged antibody probes used in this study.

Sample	Antibody target	Calculated *m/z* of antibody mass-tag $[M+H]^+$
Colorectal cancer	Actin-αSM (α-Smooth Muscle Actin)	1251.675
	CA9	1178.595
	CD4	1293.743
	CD20	997.521
	CD44	1102.579
	CD68	1216.741
	CD8α	1350.764
	Collagen-1A1 (COL1A1)	1234.861
	Histone H2A.X	1226.811
	Ki67	1320.754
	Na/K ATPase-α1	1222.791
	PanCK (Pan-Cytokeratin)	1288.708
	PTEN	1132.590
	VIM (Vimentin)	1230.841
Mouse brain	GFAP	1011.537
	GLUT1 (SLC2A1)	856.548
	MBP (Myelin Basic Protein)	1365.717
	NeuN	1308.696
	NF-L (Neurofilament Light)	1345.730
	SYN-I (Synapsin I)	1482.760

Preparations

Using standard protocols, low resolution reference images of all slides were taken on an Epson Perfection V850 Pro flatbed scanner before matrix application. Slides for lipid imaging were sprayed with 2,5-dihydroxybenzoic acid (DHB) matrix for positive ion mode measurement and with *N*-(1-naphthyl)ethylenediamine dihydrochloride (NEDC) matrix for negative ion mode measurements using Bruker standard methods on a M3+ or TM-sprayer (HTX Technologies, Chapel Hill, NC, USA). An on-tissue trypsin digestion was performed on a colorectal cancer section using standard protocols described in the literature [1]. On-tissue PNGase digestion to release N-glycans was conducted using the basic N-glycan Imaging Kit according to the instructions provided by Glycopath (Charleston, SC, USA). MALDI HiPLEX-IHC experiments were performed according to the standard protocol described by AmberGen [2]. The photocleavable mass-tags from AmberGen that were used are listed in Table 1. Slides for peptide imaging, N-glycan imaging, or MALDI HiPLEX-IHC were sprayed with α-cyano-4-hydroxycinnamic acid (HCCA) using a M3+ sprayer and a Bruker standard method. Lastly, red phosphorus was spotted in the upper right corner of the IntelliSlides® for mass calibration purposes.

Data acquisition

All MALDI Imaging measurements were performed on the neofleX Imaging Profiler using default application methods. The main method parameters are listed in Table 2. All data were acquired in positive reflector mode with 20 µm raster width and a laser frequency of 10,000 Hz if not noted otherwise. To ensure a stable total ion current (TIC), the new image stabilization feature was switched on for all measurements. Initial external calibration was conducted on a red phosphorus calibrant. The data were acquired with flexCompass 2025 containing, among other software applications, flexControl and flexImaging. The SCiLS™ autopilot workflow was used for automated measurement setup including checkpoints for correct laser focus tuning, alignment and detector gain control. Trypsin autolysis peaks or *m/z*-values of the antibody mass-tags were used as a reference mass list when an online calibration was performed, in the case of peptide imaging or MALDI HiPLEX-IHC experiments.

Table 2. Main method parameters used for the different experiments.

	Experiment				
	Lipid imaging positive ion mode	**Lipid imaging negative ion mode**	**Peptide imaging**	**N-glycan imaging**	**MALDI HiPLEX-IHC**
Laser shot count	126	126	128	180	256
Mass range [*m/z*]	500-1100	500-1200	800-2500	900-4000	800-1200
Online calibration function	off	off	on	off	on

Histological staining and digital microscopy

Sections were stained with hematoxylin and eosin (H&E) using standard procedures after MALDI Imaging data acquisitions. High-resolution microscopy images were acquired on a Hamamatsu NanoZoomer SQ digital slide scanner using the 20x or 40x magnification.

Software

The software SCiLS Lab 2024b was used for visualization and statistical analysis of MALDI Imaging data. MALDI HiPLEX-IHC data was additionally visualized in the novel viewer SCiLS Scope 1.0. Delta *m/z* diagrams were created with Origin 2022 SR1 (OriginLab Corporation). FlexAnalysis 4.2 was used to calculate the mass resolution of single spectra from MALDI Imaging datasets.

Results

Signal stability over time and use is a key enabler for unbiased images. Post acquisition normalization is widespread but suffers principal limitation when signals approach the limit of detection (LOD). Signal stabilization via ultrastable detectors, stabilized laser power and gain control to counter matrix effects is a fundamental different approach preventing compromised image data. A novel image stabilization feature in the acquisition software compensates for potential fading effects enabling high signal stability over time in combination with enhanced imaging detectors.

MALDI Imaging of lipids in positive and negative ion mode

Signal stability and robustness of the neofleX Imaging Profiler during MALDI Imaging data acquisitions of lipids were tested in three consecutive runs of two sections per run of a rat brain sample. All six datasets were imported into one SCiLS Lab file and an unsupervised segmentation analysis was performed.

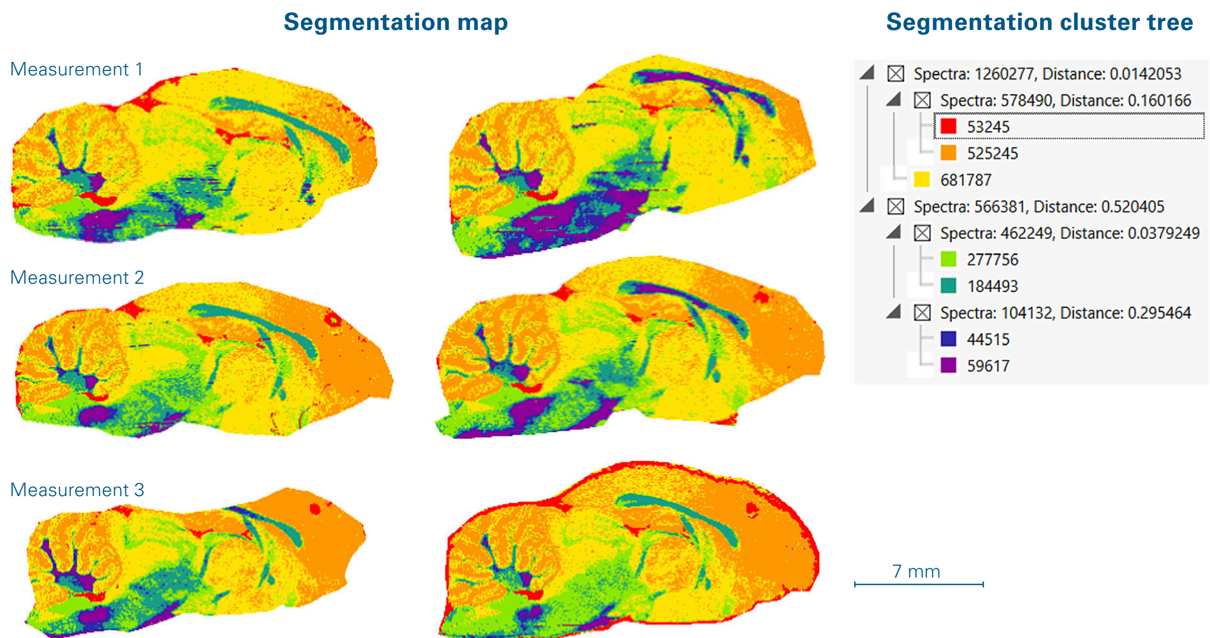

Figure 1
Positive ion mode lipid imaging on the neofleX Imaging Profiler.
Segmentation analysis was performed on the combined dataset containing all three measurements to highlight reproducibility.

The automatic segmentation split is between tissue regions and not between samples as demonstrated for three independent measurements with two samples per acquisition (Figure 1). This indicates high reproducibility over a total of 1 million pixels. A mass resolution of more than 10,000 was determined for six lipids ranging from *m/z* 740-850 commonly present in positive ion mode lipid imaging data. A similar result was obtained in a negative ion mode lipid imaging experiment. Segmentation analysis of three serial measurements of rat brain sections demonstrated high robustness as spectral variability between different tissue regions was higher compared to the variability between measurements (Figure 2A). A total ion count (TIC) image over the three datasets showed that signals were stable over a total of thirteen measurement hours without showing any sign of fading (Figure 2B). The constant TIC

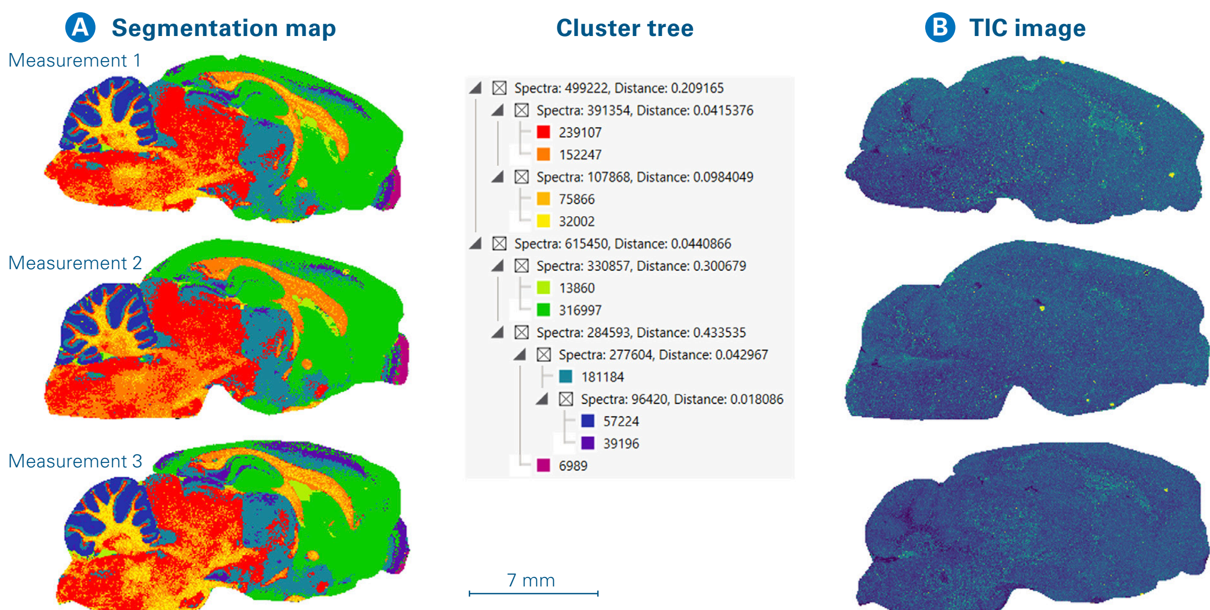

Figure 2
Negative ion mode lipid imaging on the neofleX Imaging Profiler.

across the measurement nicely illustrates the impact of the build in gain stabilization features (enhanced detector, optimized laser parameter, TIC control) enabling unbiased and constant LOD measurements.

MALDI HiPLEX-IHC

MALDI HiPLEX-IHC is a spatial biology workflow enabling multiplexed targeted protein expression analysis in tissues powered by photocleavable mass-tagged antibody probes from AmberGen, Inc. (Billerica, MA, USA). The neofleX software suite supports automated data acquisition of MALDI HiPLEX-IHC samples and generation of an OME-TIFF file allowing to visualize the spatial proteomics result in the viewer SCiLS Scope (see TechNote TN-59 for more information on SCiLS Scope). Further data analysis strategies are supported in SCiLS Lab, such as co-registration of microscopy images and statistical analysis.

To test the analytical performance of the neofleX Imaging Profiler for MALDI HiPLEX-IHC, data were analyzed with respect of potential mass shifts during an imaging run. The mass stability in terms of accuracy was significantly improved when applying the online calibration function. Mass shifts of 300 ppm were determined without online calibration (Figure 3A). With online calibration, the mass stability was improved 10fold to 30 ppm (Figure 3B).

Finally, the expression of fourteen target proteins were examined in a colorectal cancer sample with the MALDI HiPLEX-IHC. Figure 4 reflects the expression of five selected proteins including structural cell components such as actin-alpha smooth muscle, vimentin, a nuclear marker (histone 2AX), a tumor marker (PanCK), and a macrophage marker (CD68).

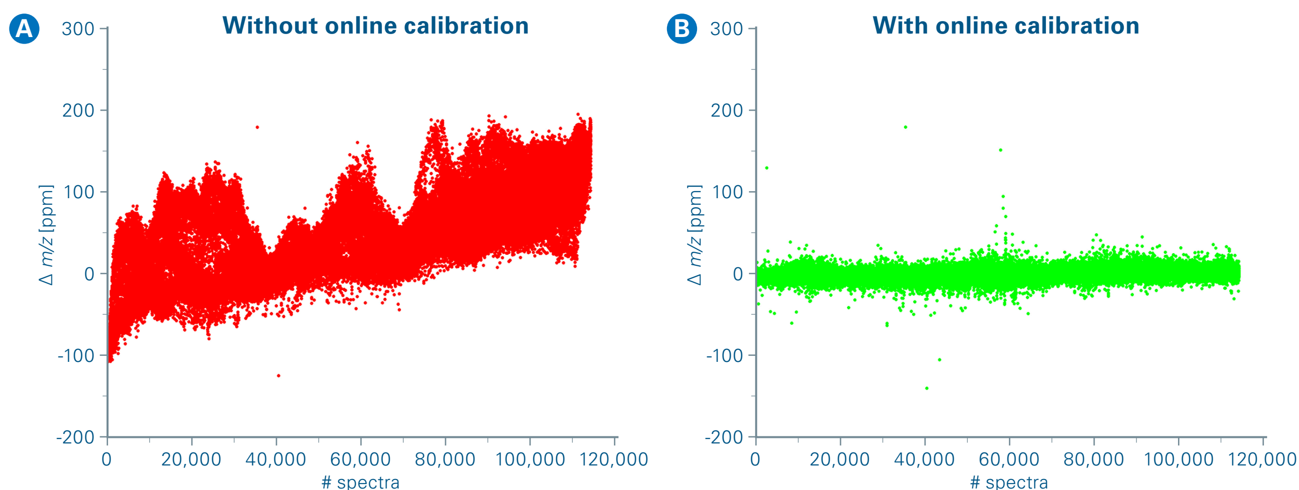

Figure 3

The online calibration function improves the mass stability over time.

MALDI HiPLEX-IHC Imaging data was plotted without (A) and with (B) online calibration shown for the vimentin mass tag at *m/z* 1320.754. Without online calibration, the mass shifted from –100 to 200 ppm (A). With online calibration, the 90% and 10% percentile for all spectra was within ±30 ppm.

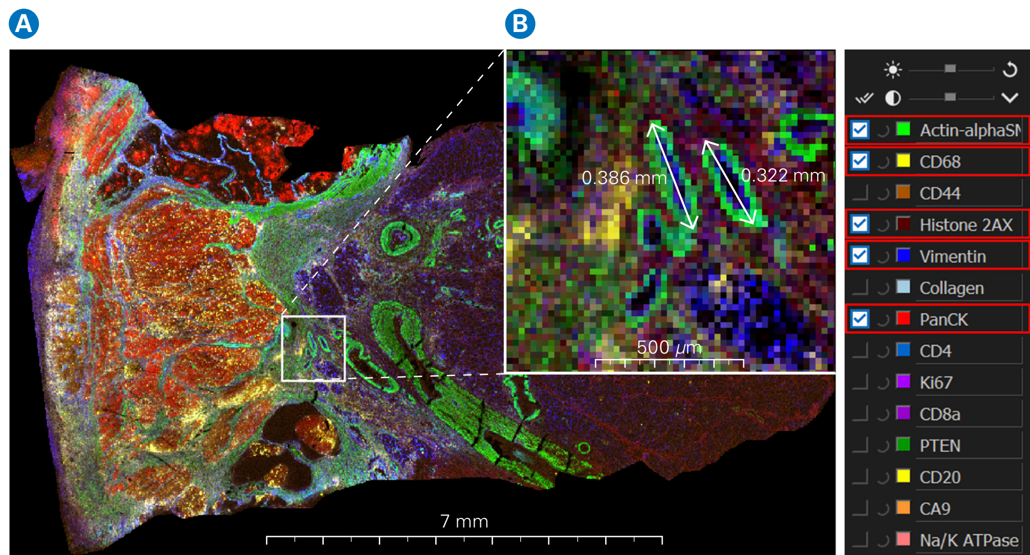

Figure 4

Visualization of MALDI HiPLEX-IHC data from a colorectal cancer sample in SCiLS Scope.

Five different channels, specific for the target proteins indicated in the legend on the right, were activated and the distribution of the proteins is shown (A). In the zoomed window (B) the new SCiLS Scope tool for measuring distances was applied.

MALDI Imaging of N-glycans

The analysis of released N-glycans by MALDI Imaging is an established method which is of high relevance in cancer research, as most known cancer biomarkers are glycoproteins or other glycoconjugates. N-glycan imaging was performed on a serial section of the same colorectal cancer specimen used for peptide imaging. Glycan compositions, and therefore masses, are highly conserved across cancer types. Importantly, the observation of about a 100 N-glycan masses which were matched with a Bruker N-glycan database containing 165 entries, demonstrates the depth of information accessible with this approach. The ion images of six known N-glycans were visualized in SCiLS Lab by artificial color coding as indicated in the bars above each putative glycan (Figure 5). The glycans appear with high intensities in specific tissue

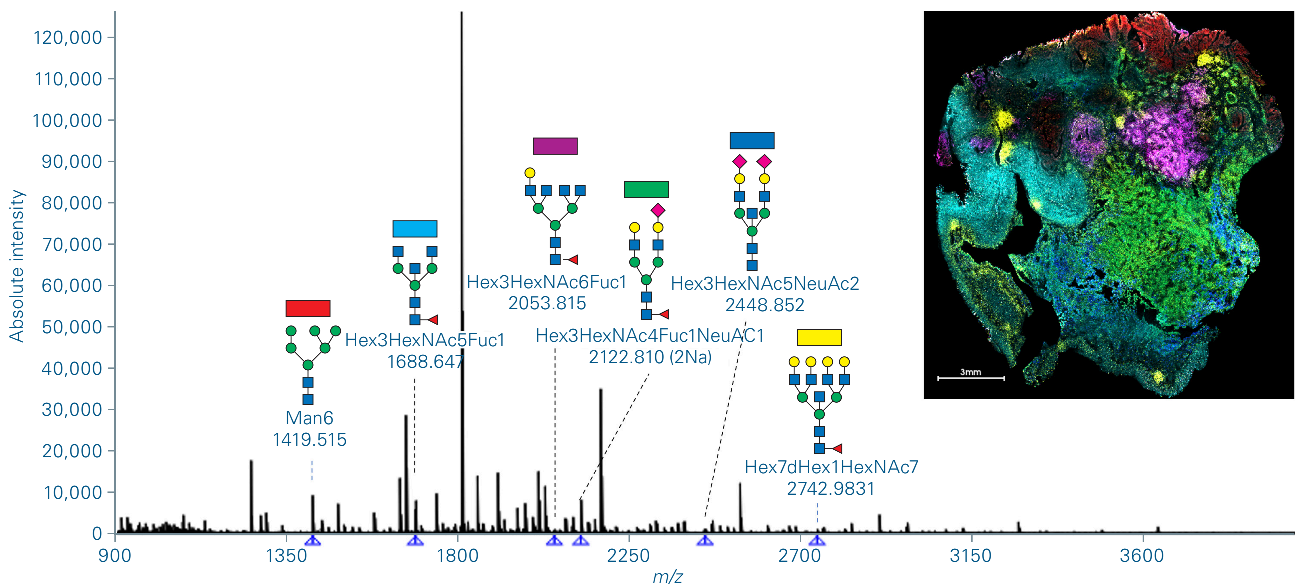

Figure 5

Mean spectrum of the entire N-glycan imaging dataset from a colorectal cancer section and multichannel ion image showing the distribution of the indicated peaks (blue triangles under the x-axis).

Putative structures of the N-glycans are given above each peak. The colors used for false-color coding of the ion image are given above each glycan structure. Sample courtesy of A. Tannapfel and Jens Christmann, Institute of Pathology, University of Bochum, Germany.

regions. Distinct glycans, such as high-mannose glycans known to be present on the surface of tumor cells, are visualized with high intensities in specific regions of tumors [3].

As for other MALDI Imaging applications, comparing ion intensity distribution with tissue morphology is also crucial for released N-glycan imaging. Therefore, a high-resolution microscopy image of the same tissue section was imported into the SCiLS Lab dataset and superimposed on the glycan ion distribution (Figure 6). A tetraantennary N-glycan, *m/z* 2053.815, was present primarily in the mucinous tumor region of a colon cancer while bisecting *N*-acetylglucosamine glycans such as *m/z* 1688.647 occurred mainly in the epithelial crypt cell region as previously described by Drake et al. [3]. Two other glycans at *m/z* 2122.810 and 2448.852 were primarily present in the tumor stroma region. A complex fucosylated N-glycan (*m/z* 2742.983) was colocalized with a MALDI HiPLEX-IHC image of CD20, which is a marker for B-cells.

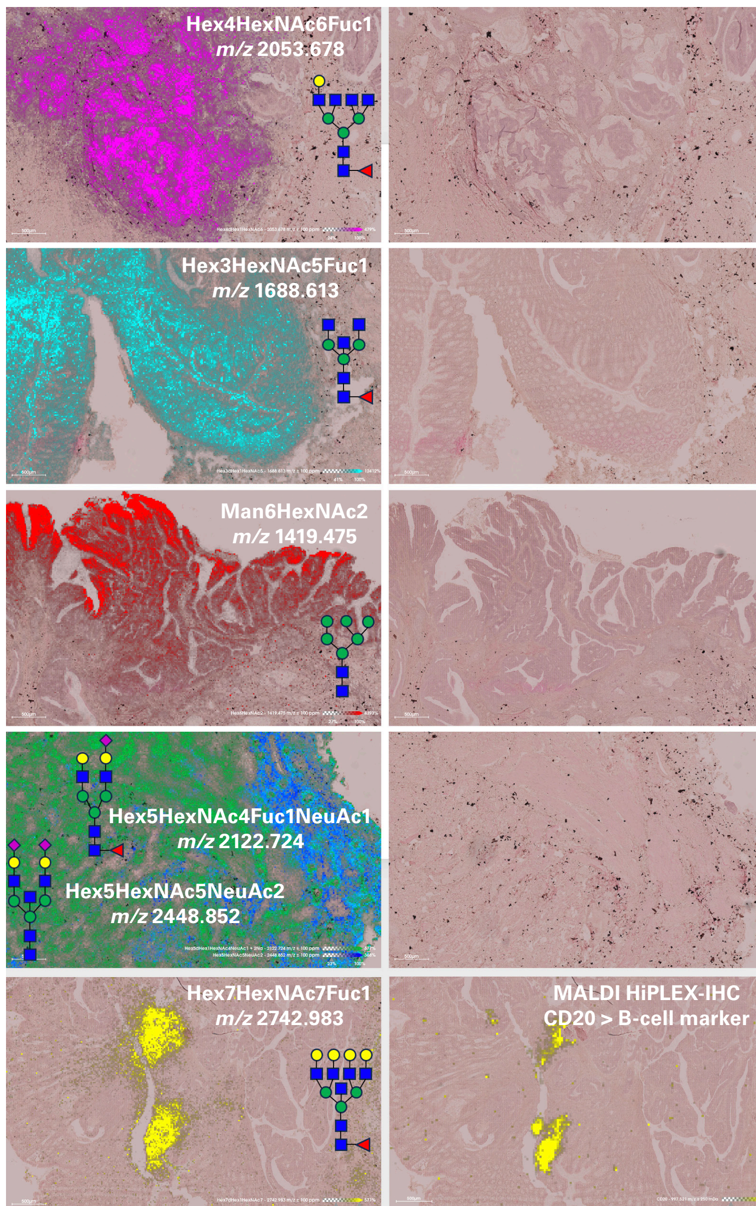

Figure 6

N-glycan ion images superimposed on the tissue morphology (H&E image) of a colorectal cancer tissue section (left panels).

Putative glycan compositions are provided for each *m/z*-value, for which the ion distribution is shown by false-color coding. Low intensity pixels were made transparent. The right panels show the respective H&E image without MALDI Imaging information except for the bottom right image indicating the distribution of a CD20 mass-tag from a subsequent MALDI HiPLEX experiment with 30 μm raster width on the same sample. Samples were generously provided by A. Tannapfel and Jens Christmann, Institute of Pathology, University of Bochum, Germany.

Conclusion

- The neofleX Imaging Profiler showed excellent performance for various MALDI Imaging applications such as targeted protein, N-glycan, and lipid imaging.
- MALDI HiPLEX-IHC experiments were successfully conducted using automated processes and easy viewing in SCiLS Scope resulting in images of expressed protein landscapes with large field-of-view to complement spatial biology methods.
- The neofleX software suite supports automatic measurement setups with the SCiLS autopilot for reliable acquisitions regardless of user experience.

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