**Light sheet fluorescence microscopy guided MALDI-imaging mass spectrometry of cleared tissue samples**

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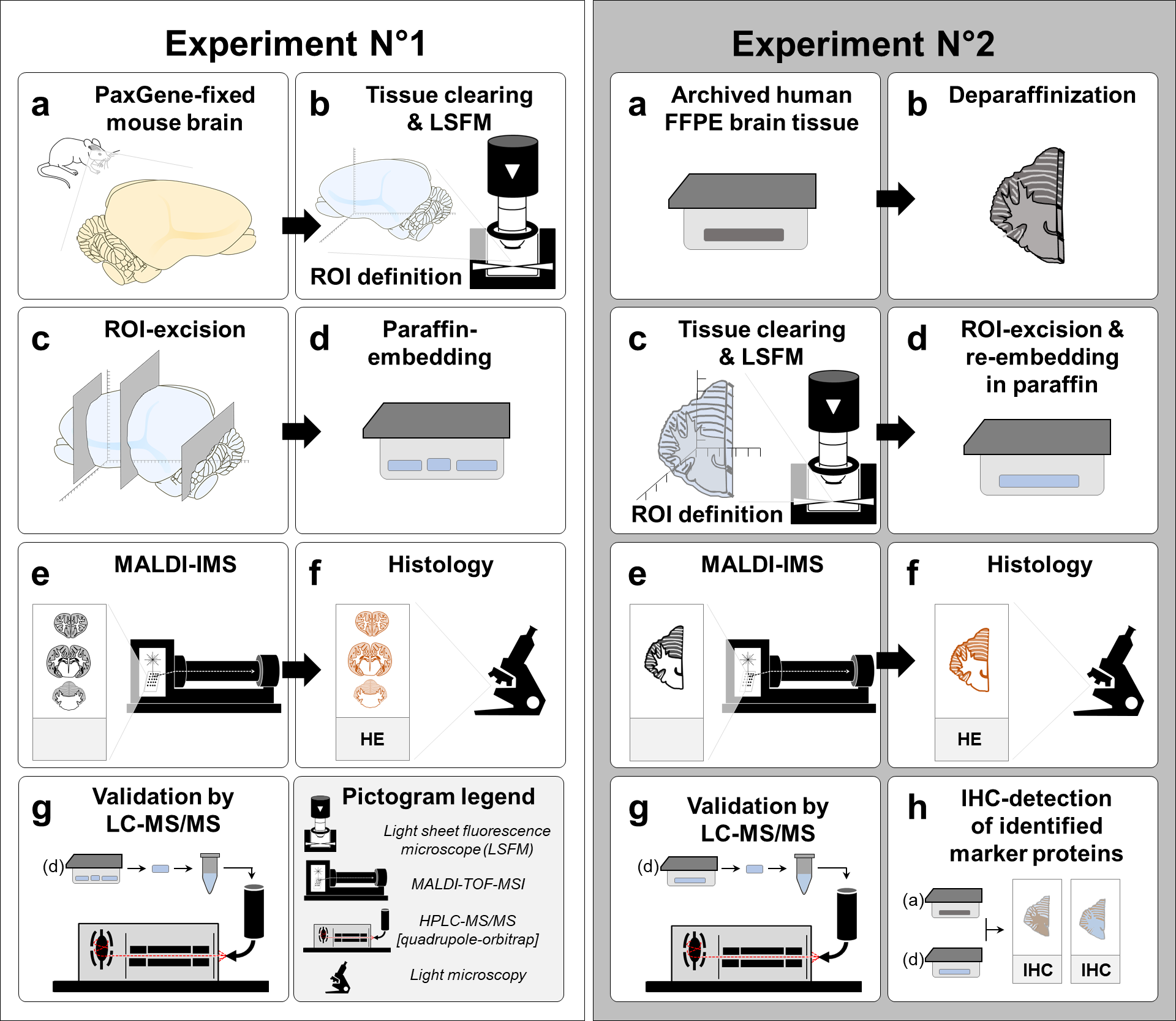
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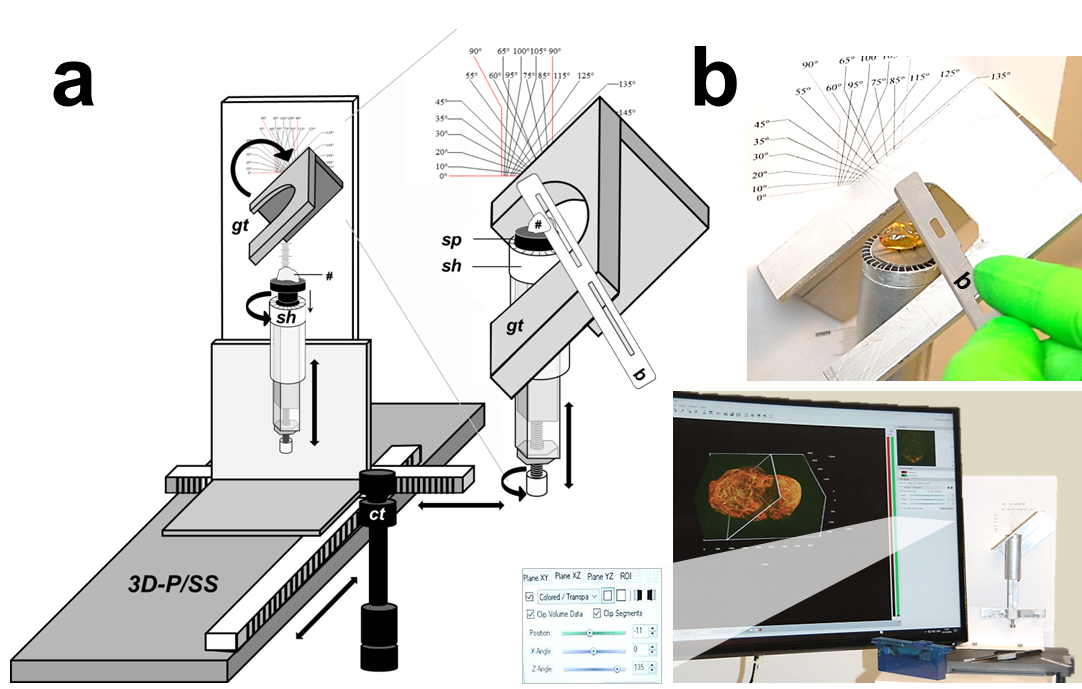
**Supplemental data**

**Supplemental Figure 1.** **Schematic experimental design and work-flow of Experiment N°1 and Experiment N°2.**

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**Supplemental Figure 1.** **Schematic experimental designs and work-flows.** To demonstrate the feasibility of MALDI-MSI on paraffin sections of optically cleared tissue samples, two experiments were performed. In **Experiment N°1**, a freshly dissected mouse brain was fixed in formaldehyde-free fixation solution (PaxGene) (**a**), optically cleared (3DISCO) and subjected to light sheet fluorescence microscopy (LSFM) (**b**). Regions of interest (ROIs) defined by LSFM were dissected from the cleared tissue (**c**), and embedded in paraffin (**d**). Paraffin sections were subjected to MALDI-MSI (**e**) and subsequent HE-staining (**f**).For validation of MALDI-MSI data, proteomic LC-MS/MS analysis was performed onadditional samples of paraffin-embedded mouse brain tissue (**g**). For **Experiment N°2**,FFPE human brain tissue (archive material, **a**) was deparaffinized, (**b**) optically cleared (3DISCO) and subjected to LSFM (**c**). Regions of interest were defined by LSFM and dissected from the cleared tissue, and re-embedded in paraffin (**d**). Paraffin sections of the re-embedded tissue were subjected to MALDI-MSI (**e**) and subsequent HE-staining (**f**). For validation of MALDI-MSI data, proteomic LC-MS/MS analysis was performed on additional samples of paraffin-embedded human brain tissue (**g**) and the spatial abundance patterns of MALDI-MSI detected tryptic peptides of different established nerve tissue marker proteins were confirmed by immunohistochemistry (**h**).

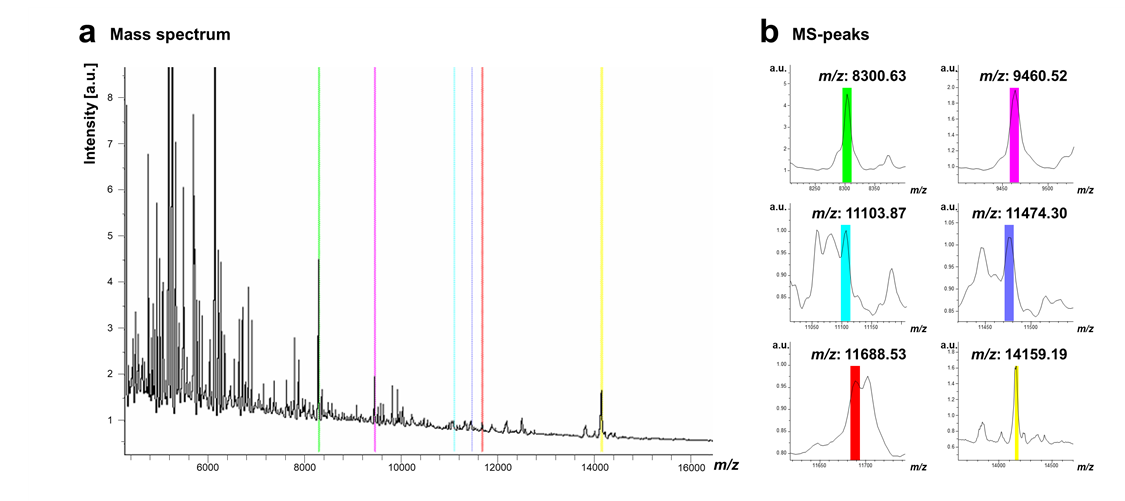
**Supplemental Figure 2**

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**Supplemental Figure 2. Excision of a LSFM-determined ROI from the cleared tissue sample.**

Within the cleared tissue, ROI(s) for subsequent MALDI-MSI are defined by LSFM and subsequently excised from the sample. For reasonably sized organ samples, such as complete mouse brains, with abundant distinctive anatomical details, the use of a positioning and sectioning device is dispensable and sections can be straightforwardly excised by hand, with adequate precision. However, for cutting of small-sized tissue samples, or for preparation of oblique sections through cleared samples with indistinct, uniform external shapes, the aid of a three-dimensional sample positioning device (**3D-P/SS**) with a blade guiding track (***gt***), is advantageous. Here, a self-constructed positioning and cutting-device, built from a microscope cross-table (***ct***) and few standard hardware store components is shown. **a**: Construction scheme. **b:** Gross image and detail enlargement. For accurate positioning of the sample (#) and the section plane(s), the x-, y-, and z-position of the sample holder (***sh***) can be precisely be adjusted, the sample-plate (***sp***) and the blade guiding track can be rotated by 360°. To precisely replicate the orientation of LSFM optical section plane(s), the sample plate with the tissue sample (***#***) is removed from the sample chamber of the LSFM and directly transferred to the sample plate holder of the 3D-positioning/cutting device. The sample-positioning data and the section plane angle(s) of the LSFM-viewer software are correspondingly transferred to the gadget, and the cleared tissue sample is sectioned, using a microtome blade (***b***). Care should be taken to place the physical section plane through the cleared tissue sample approximately 0.5 mm parallel to the optical LSFM-section plane, to avoid cutting directly through the structure of interest. In the present example, the positioning of a cleared mouse brain for preparation of an 135° oblique frontal section through the cerebrum and the diencephalon is shown (the presented example intends to illustrate the sectioning process, it does not show the same ROI-section plane orientation examined in the MALDI-MSI experiment). Note that the described method of tissue-sectioning works well only for solid, shape-retaining optically cleared tissue samples, but not for soft/gelatinous samples.

**Supplemental Figure 3**

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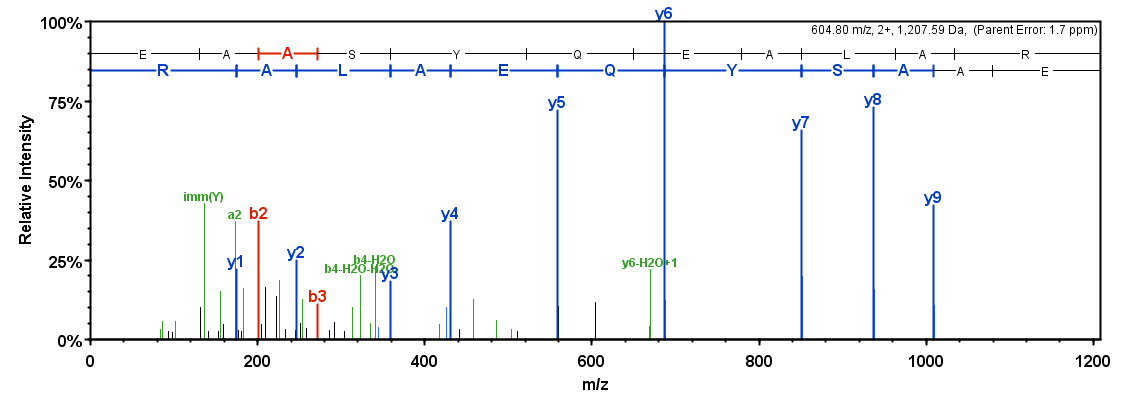
|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **c**  **Protein** | **Protein accession numbers** | **Protein molecular weight (Da)** | **Protein identification probability** | **Exclusive unique peptide count** | **Sequence coverage (%)** | **Corresponding MALDI-MSI Peak (*m/z*)** |
| GNG3 | P63216|GBG3\_MOUSE | 8304.90 | 100.00% | 2 | 60.00% | 8300.63 |
| RPS27 | Q6ZWU9|RS27\_MOUSE | 9460.90 | 100.00% | 2 | 28.60% | 9460.52 |
| MRPS36 | Q9CQX8|RT36\_MOUSE | 11101.10 | 100.00% | 4 | 57.80% | 11103.87 |
| RPLP1 | P47955|RLA1\_MOUSE | 11475.00 | 100.00% | 2 | 51.80% | 11474.30 |
| NDUFB3 | Q9CQZ6|NDUB3\_MOUSE | 11692.30 | 99.10% | 2 | 14.40% | 11688.53 |
| SCOC | Q78YZ6|SCOC\_MOUSE | 14155.00 | 100.00% | 2 | 35.20% | 14159.19 |

**Supplemental Figure 3. MS-spectra of MALDI-IMS and identified proteins validated by LC-MS/MS analysis in experiment N°1 (PaxGene-fixed, 3DISCO-cleared, paraffin-embedded mouse brain tissue).** **a**: MALDI-mass spectrum from 4-16 kDa. MS-protein peaks of six identified masses are highlighted in different colors. **b**: Detail enlargements of these mass spectra peaks with corresponding *m/z* values. The six featured mass spectra correspond to ions of the same LC-MS/MS-confirmed proteins shown in Figure 1. *m/z*: 8300.63: GNG3 (guanine nucleotide-binding protein subunit gamma-3); *m/z*: 9460.52: RPS27 (40S ribosomal protein S27); *m/z*: 11103.87: MRPS36 (28S ribosomal protein S36, mitochondrial); *m/z*: 11474.30: RPLP1 (60S acidic ribosomal protein P1); *m/z*: 11688.53: NDUFB3 (NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 3); *m/z*: 14159.19: SCOC (Short coiled-coil protein). Relevant parameters of the LC-MS/MS protein identification are provided in table c.

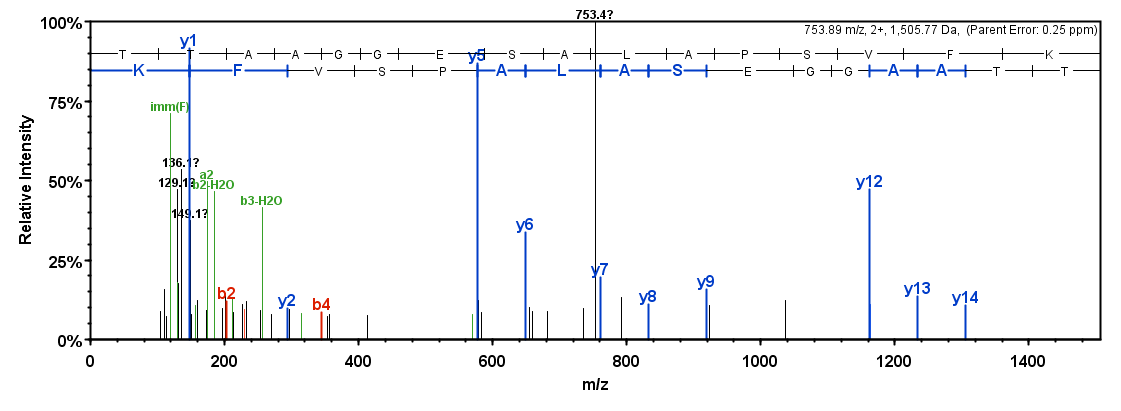
**Supplemental Figure 4**

**a**

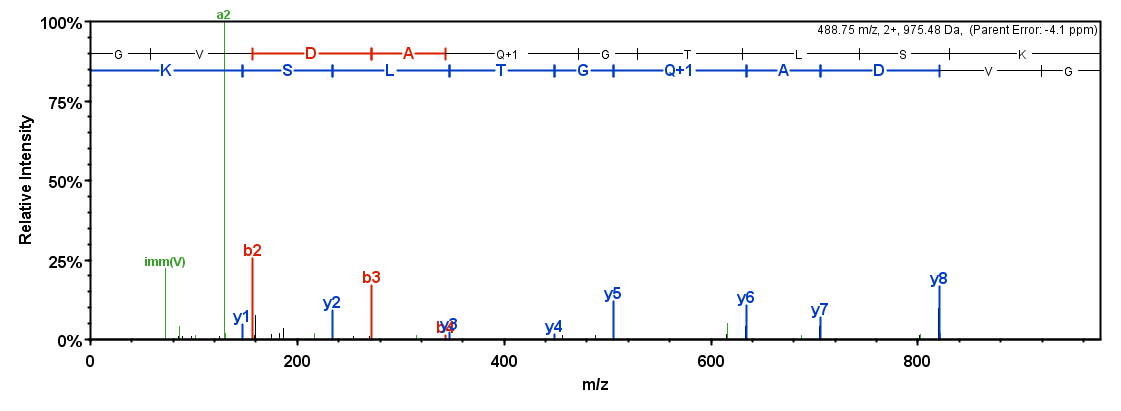
**GFAP (*m/z* 1208.83, EAASYQEALAR + H+)**



**MAP2 (*m/z* 1506.91, TTAAGGESALAPSVFK + H+)**

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**MBP (*m/z* 975.63, GVDAQGTLSK + H+)**

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**b**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Protein | Protein accession numbers | Protein molecular weight (Da) | Protein identification probability | Unique peptide count | Peptide sequence | Peptide identification probability | Calculated +1H Peptide Mass |
| GFAP | P14136|GFAP\_HUMAN | 49881.40 | 100.00% | 48 | EAASYQEALAR | 99.70% | 1208.59 |
| MAP2 | P11137|MTAP2\_HUMAN | 199527.90 | 100.00% | 34 | TTAAGGESALAPSVFK | 99.70% | 1506.78 |
| MBP | P02686|MBP\_HUMAN | 33117.70 | 100.00% | 4 | GVDAQGTLSK | 99.70% | 975.5107 |

**Supplementary Figure 4. MS/MS-spectra of tryptic peptides detected by MALDI-IMS and validated by LC-MS/MS analysis in experiment N°2 (FFPE, 3DISCO-cleared, and paraffin-(re)embedded human brain tissue samples).** The MS/MS-spectra shown in (a) correspond to ions of tryptic peptides of the same established neural tissue marker proteins also detected by MALDI-MSI and confirmed by immunohistochemistry (refer to Figure 2). Tryptic peptide EAASYQEALAR+H+ (*m/z* 1208.83) corresponds to glial fibrillary acidic protein (GFAP), an astrocyte-marker. Tryptic peptide TTAAGGESALAPSVFK+H+ (*m/z* 1506.91) corresponds to microtubule-associated protein 2 (MAP2), a neuronal differentiation marker.Tryptic peptide GVDAQGTLSK+H+ (*m/z* 975.63) corresponds to myelin basic protein (MBP), a marker of myelinating glia, highly abundant in the white matter. Relevant parameters of the LC-MS/MS peptide 3 identification are provided in table b.

**Supplemetal Video 1. LSFM guided MALDI-MSI** **and integration of 2D MALDI-MS images with 3D-LSFM reconstructions of organ morphology.** The video shows the 3D reconstruction of a PaxGene-fixed, optically cleared (3DISCO) mouse brain from a previous study1. Blood vessels are visualized in gray color (tagged by fluorescent labeled lectin) [0:00-0:06 sec]. The positions and orientations of the section planes of the tissue regions of interest (ROI N°1-3) selected for excision and MALDI-MSI are superimposed with MALDI-MS images subsequently acquired in paraffin sections of the corresponding locations [0:09-0:34 sec], showing the spatial distributions and intensities of guanine nucleotide-binding protein subunit gamma-3 (Gng3, *m/z*: 8300.63). Signal intensities are visualized by different colors; minimal intensities are shown in black-to blue, maximal intensities are indicated by red color (compare to the intensity-color scales shown in Fig. 1 and Fig. 2).

1. Harrison, L., et al., Fluorescent blood-brain barrier tracing shows intact leptin transport in obese mice. Int J Obes (Lond), 2018.