Supporting Information

Proteoform-Selective Imaging of Tissues Using Mass Spectrometry

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Supporting Information

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Experimental Section

I. Reagents and Materials

HPLC-MS grade water was purchased from MilliporeSigma (Burlington, MA). HPLC-MS grade acetonitrile (ACN) was bought from Fisher chemical (Hampton, NH), 99.8+% chloroform was purchased from Alfa Aesar (Tewksbury, MA). 100% acetic acid (AA) was purchased from Honeywell (Charlotte, NC). Ethanol 200 Proof was from Decon Laboratories, INC (King of Prussia, PA). The working solvent used in this study was ACN/H2O/AA (65/34/1, v/v/v). Two standards were added to the solvent at a 0.15 µM (the first replicate), 0.3 µM (the second replicate) and 0.5 µM (the third replicate) concentration to monitor the stability and magnitude of the signal during the experiment. The standards included lysozyme from hen egg white and bradykinin acetate salt. Both standards were purchased from Sigma-Aldrich (Burlington, MA). Polymicro flexible fused silica capillary tubing (OD 150µm, ID 50 µm and OD 790µm, ID 200µm) were purchased from Molex (Thief river falls, MN).

II. Animal Tissues

Sprague Dawley axial rat brain tissue sections were provided by Dr. Elizabeth Neumann from Vanderbilt University. Snap frozen Sprague Dawley rat brain was purchased from BioIVT (Westbury, NY) and stored at −80 °C. Brain tissues were fixed to the object holder using optimal cutting temperature compound (OCT compound). To avoid contamination and signal suppression, only a small amount of OCT compound was used to mount the tissue block and care was taken to avoid any contact between OCT and the tissue sections used for nano-DESI MSI analysis. The tissues were sectioned at −21 °C to a 10µm thickness using a CM1850 Cryostat (Leica Microsystems, Wetzlar, Germany). Tissue sections were thaw mounted onto glass microscope slides (IMEB, Inc Tek-Select Gold Series Microscope Slides, Clear Glass, Positive Charged). Mouse brain sections were stored in −80 °C freezer before nano-DESI MSI analysis.

III. Nano-DESI Mass Spectrometry imaging

For protein imaging, brain tissue sections were delipidated by immersing them successively in 70%, 90% 100% ethanol solutions for 20s each, and 99.8% chloroform for 25s right before nano-DESI imaging experiments.[1] This de-lipidation step removes lipids from tissue sections to prevent suppression of protein signals. In addition, it is generally accepted that during ethanol wash, proteins precipitate in situ and stay localized.[2] Imaging experiments were performed on a Q-Exactive HF-X Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) equipped with custom-designed nano-DESI source. The nano-DESI probe comprises the primary and nanospray fused silica capillaries (OD 150µm, ID 50µm) positioned at a 90-degree angle. The probe is positioned in front of the mass spectrometer inlet. A photo of the nano-DESI source is shown in Figure S1. The solvent is propelled through the primary capillary using a syringe pump at 0.5µL/min. The analytes are extracted into the liquid bridge formed between the capillaries and sample surface. Extracted analytes are transferred through the nanospray capillary and electro-sprayed at the mass spectrometer inlet. A third silica fused capillary (OD 790µm, ID 200µm) pulled to ~20 µm OD serves as a shear force probe. Two piezoelectric ceramic plates are attached to the probe: one is placed ~1cm above the tip and another one placed ~2cm above the first one. The shear force probe is positioned a few micrometers in front of the primary and nanospray capillary in the direction of scanning to maintain a constant distance between the nano-DESI probe and the surface. Details of the nano-DESI source setup can be found in our previous publications.[3][4] The scanning velocity was set to 0.04 mm/s and the strip step was set to 150 µm.

All the experiments were performed in positive ionization mode. The tuning and calibration of the mass spectrometer were performed using the Pierce™ LTQ Velos ESI positive ion calibration solution (Thermo Scientific). The mass range was set to m/z 400-1800 to exclude solvent peaks but include peaks corresponding to both standards and endogenous proteins. The spray voltage was set to 4.0 kV with in-source fragmentation of 14ev to enhance protein signals. The inlet temperature was 300°C. The ion-funnel RF level was set to 100%. The mass resolution was 120,000 (m/∆m at m/z 200). The automatically gain control (AGC) target was 10⁶, the maximum injection time was 200 ms, and 2 microscans (the first and third replicates) and 4 microscans (the second replicate) were used to improve the signal-to-noise ratio at a minimum loss of the spatial resolution.
IV. On-tissue Top-Down Proteomics

Targeted MS/MS experiments were performed on a tissue section adjacent to the imaged section using higher-energy collisional dissociation (HCD). Four inclusion lists provided m/z values and the corresponding charge states of protein signals observed in four regions on the brain tissue: white matter, grey matter, blood vessel, and hippocampal formation region. MS/MS was performed by scanning the nano-DESI probe over a small region of the above-mentioned regions and collecting fragmentation spectra for protein peaks enhanced in a specific region. The scanning velocity was 0.02 mm/s. The mass resolution was 60,000 at m/z 200. The isolation window was 3 m/z, while 0.5 m/z isolation window was used for the species which are overlapped with other species within 3 m/z. HCD spectra were acquired at three values of the normalized collision energy (NCE) of 23, 25, 30. The AGC was 10⁶ and the maximum injection time was 800 ms.

V. Ion image generation

Ion images were generated using a custom Python script developed at Purdue University (https://github.com/hanghu1024/RAW-MSI-generator). This script uses the pymsfilereader package to extract data from .RAW files. Mass spectral data were split using scan filters and aligned according to scan times. A mass window of 10 ppm was used to extract signals of selected m/z values, which were used to generate ion images. All the signals were normalized to the corresponding total ion current (TIC) to compensate for the signal variation during the MSI experiment.

VI. On-tissue top-down proteomics data analysis

The .RAW files were processed using ProSightPD 4.0 for Proteome Discoverer 2.5 (PSPD). The PSPD Comprehensive Discovery Proteomics with FDR analysis template was used. That workflow contains three search nodes: an annotated proteoform search with a 2.2 Da precursor tolerance, an annotated proteoform search with a 100 Da precursor tolerance, and a subsequence search with a 10 ppm precursor tolerance. All three search nodes used a Rattus Norvegicus (Rat: Taxon 10116) database created from UniProt release 2020_04. The workflow also performed a false discovery rate (FDR) calculation. Results that passed the 1% FDR cutoff were annotated using PFR Accessions and included in the output files (pdResult and tdReport). TDViewer (http://tdviewer2.northwestern.edu) was used to review the results in the tdReport.
Figure S1. The custom nano-DESI source coupled to a Q-Exactive HF-X Orbitrap mass spectrometer. 1- primary capillary; 2- nanospray capillary; 3- shear force sensor; 4- rat brain tissue section; 5- mass spectrometer inlet.

Figure S2. An average mass spectrum obtained for the hippocampal region highlighted in the optical image. The spectrum is dominated by different charge states of ubiquitin marked with green circles.
Figure S3. Optical images of an axial mouse brain tissue section before (a) and after (b) nano-DESI MSI analysis. Ion images of the intact proteins normalized to TIC: (c) m/z 936.613517+, 15,905 Da, hemoglobin subunit beta-1 (HBB-1) or hemoglobin subunit beta-2 (HBB-2), (d) m/z 786.8497+, 5,501 Da, unknown, (e) m/z 753.733689+, 6,775 Da, unknown, (f) m/z 627.240989+, 5,636 Da, ATP synthase subunit epsilon, mitochondrial, unmodified, (g) m/z 859.128910+, 7,723 Da, unknown, (h) m/z 813.157510+, 8,122 Da, ATP synthase subunit e, mitochondrial, unmodified, (i) m/z 952.638789+, 8,565 Da, polyubiquitin, unmodified, (j) m/z 1027.119310+, 10,261 Da, dynein lightchain 2, cytoplasmic, acetylation, (k) m/z 631.333519+, 11,976 Da, unknown, (l) m/z 812.520611+, 8,927 Da, ATP-synthase-coupling factor 6, mitochondrial, unmodified. Scale bar: 3mm.
Figure S4. Replicate 1: (a) An optical image of an axial mouse brain tissue section before nano-DESI MSI analysis. Ion images of the intact proteins normalized to TIC. (b) m/z 894.938417+, 15,197 Da, hemoglobin subunit alpha (HBA1), unmodified, (c) m/z 992.28116+, 15,861 Da, hemoglobin subunit beta-1 (HBB-1), R104 di-methylation, (d) m/z 936.613517+, 15,905 Da, hemoglobin subunit beta-1 (HBB-1) or hemoglobin subunit beta-2 (HBB-2), (e) m/z 741.809419+, 14,075 Da, myelin basic protein (MBP), unmodified, (f) m/z 682.357227+, 18,397 Da, myelin basic protein (MBP), N-terminal acetylation, (g) m/z 765.406713+, 9,937 Da, acyl-CoA-binding protein (ACBP), acetylation, (h) m/z 786.8497+, 5,501 Da, unknown, (i) m/z 784.5647+, 5,485 Da, cytochrome c oxidase subunit 7c, mitochondrial, (COX7c), unmodified, (j) m/z 859.1289+, 7,723 Da, unknown, (k) m/z 709.937510+, 4,963 Da, thymosin beta-4, acetylation, (l) m/z 753.73369+, 6,775 Da, unknown, (m) m/z 627.2409+, 5,636 Da, ATP synthase subunit epsilon, mitochondrial, unmodified, (n) m/z 998.794710+, 9,978 Da, cytochrome c oxidase subunit 6B1 (COX6B1), N-terminal acetylation, (o) m/z 813.157510+, 8,122 Da, ATP synthase subunit e, mitochondrial, unmodified, (p) m/z 952.63879+, 8,565 Da, thymosin beta-4, polyubiquitin, unmodified, (q) m/z 1027.119310+, 10,261 Da, dynein light chain 2, cytoplasmic, acetylation, (r) m/z 631.333519+, 11,976 Da, unknown, (s) m/z 812.520611+, 8,927 Da, ATP synthase–coupling factor 6, mitochondrial, unmodified, (t) m/z 679.2137+, 4,747 Da, thymosin beta-4, acetylation. Scale bar: 3mm.
Figure S5. Replicate 2: (a) An optical image of an axial mouse brain tissue section before nano-DESI MSI analysis. Ion images of the intact proteins normalized to TIC. (b) m/z 894.938317+, 15,197 Da, hemoglobin subunit alpha (HBA1), unmodified, (c) m/z 992.345316+, 15,861 Da, hemoglobin subunit beta-1 (HBB-1), R104 di-methylation, (d) m/z 936.613117+, 15,905 Da, hemoglobin subunit beta-1 (HBB1) or hemoglobin subunit beta-2 (HBB-2), (e) m/z 741.915919+, 14,075Da, myelin basic protein (MBP), unmodified, (f) m/z 682.37527+, 18,397 Da, myelin basic protein (MBP), N-terminal acetylation, (g) m/z 765.406713+, 9,937 Da, acyl-CoA-binding protein (ACBP), acetylation, (h) m/z 812.517611+, 8,927 Da, ATP synthase–coupling factor 6, mitochondrial, unmodified, (i) m/z 784.5647+, 5,485 Da, cytochrome c oxidase subunit 7c, mitochondrial (COX7c), unmodified, (j) m/z 709.93857+, 4,963 Da, thymosin beta-4, acetylation, (k) m/z 753.73679+, 6,775 Da, unknown, (l) m/z 627.13029+, 5,636 Da, ATP synthase subunit epsilon, mitochondrial, unmodified, (m) m/z 998.796910+, 9,978 Da, cytochrome c oxidase subunit 6B1 (COX6B1), N-terminal acetylation, (n) m/z 813.256310+, 8,122 Da, ATP synthase subunit e, mitochondrial, unmodified, (o) m/z 952.63849+, 8,565 Da, polyubiquitin, unmodified, (p) m/z 1026.91810+ 10,261 Da, dynein lightchain 2, cytoplasmic, acetylation, (q) m/z 631.333519+, 11,976 Da, unknown. Scale bar: 3mm.
Figure S6. A zoomed-in region of the mass spectrum showing the +19 charge state of the 14.1 kDa MBP proteoforms and +25 charge state of the 18.4 kDa MBP proteoforms.

| PTM                                | Signal Intensity [x10^6] |
|------------------------------------|--------------------------|
| unmodified                         |                           |
| N-terminal acetylation             |                           |
| N-terminal acetylation, methionine sulfoxide |          |
| phosphorylation                    |                           |
| N-terminal acetylation, phosphorylation |                     |
| N-terminal acetylation             |                           |

| Mass /Da | 14,075 | 14,121 | 14,136 | 14,151 | 14,200 | 18,397 |
Figure S7. MBP proteoforms: (a) An optical image of an axial mouse brain tissue section after nano-DESI MSI analysis. Ion images of the +19 charge state of the 14.1 kDa MBP proteoforms. Top panels show ion images normalized to the TIC. Bottom panels show images generated by plotting the ratio of the individual proteoform signal to the sum of signals of all the MBP proteoforms: (b) m/z 741.809419+, 14,075 Da, unmodified, (c) m/z 742.652819+, 14,091 Da, (d) m/z 743.388719+, 14,105 Da, (e) m/z 744.233519+, 14,121 Da, N-terminal acetylation, (f) m/z 745.023519+, 14,136 Da, N-terminal acetylation, methionine sulfoxide, (g) m/z 745.813219+, 14,151 Da, phosphorylation, (h) m/z 747.706319+, 14,187 Da, di-methylation, phosphorylation (i) m/z 748.390119+, 14,200 Da, N-terminal acetylation, phosphorylation, (j) m/z 752.492319+, 14,278 Da, N-terminal acetylation, di-phosphorylation. (m) m/z 683.061927+, 18,412 Da, N-terminal acetylation, (n) m/z 682.357227+, 18,397 Da, N-terminal acetylation. Scale bar: 3mm.
Figure S8. MBP proteoforms: (a) An optical image of an axial mouse brain tissue section before nano-DESI MSI analysis. Ion images of the +19 charge state of the 14.1 kDa MBP proteoforms. Top panels show ion images normalized to the TIC. Bottom panels show images generated by plotting the ratio of the individual proteoform signal to the sum of signals of all the MBP proteoforms: (b) m/z 741.809419+, 14,075 Da, unmodified, (c) m/z 742.652819+, 14,091 Da, (d) m/z 744.233519+, 14,121 Da, N-terminal acetylation, (f) m/z 745.023519+, 14,136 Da, N-terminal acetylation, methionine sulfoxide, (g) m/z 745.813219+, 14,151 Da, phosphorylation, (h) m/z 747.706319+, 14,187 Da, di-methylation, phosphorylation, (i) m/z 748.390119+, 14,200 Da, N-terminal acetylation, phosphorylation, (j) m/z 749.284119+, 14,217 Da, (k) m/z 750.386719+, 14,238 Da, (l) m/z 752.492319+, 14,278 Da, N-terminal acetylation, di-phosphorylation. (m) m/z 682.357227+, 18,397 Da, N-terminal acetylation, (n) m/z 683.061927+, 18,412 Da. Scale bar: 3mm.
Figure S9. MBP proteoforms: (a) An optical image of an axial mouse brain tissue section before nano-DESI MSI analysis. Ion images of the +19 charge state of the 14.1 kDa MBP proteoforms. Top panels show ion images normalized to the TIC. Bottom panels show images generated by plotting the ratio of the individual proteoform signal to the sum of signals of all the MBP proteoforms: (b) m/z 741.859419+, 14,075 Da, unmodified, (c) m/z 742.700719+, 14,091 Da, (d) m/z 744.177819+, 14,121 Da, N-terminal acetylation, (e) m/z 744.96819+, 14,136 Da, N-terminal acetylation, methionine sulfoxide, (f) m/z 745.809519+, 14,151 Da, phosphorylation, (g) m/z 747.706319+, 14,187 Da, di-methylation, phosphorylation, (h) m/z 747.706319+, 14,187 Da, di-methylation, phosphorylation, (i) m/z 748.385319+, 14,200 Da, N-terminal acetylation, phosphorylation, (j) m/z 749.284119+, 14,217 Da, (k) m/z 750.386619+, 14,238 Da, (l) m/z 752.492319+, 14,278 Da, N-terminal acetylation, di-phosphorylation. (m) m/z 682.37527+, 18,397 Da, N-terminal acetylation, (n) m/z 682.986627+, 18,412 Da. Scale bar: 3mm.
Figure S10. Hemoglobin proteoforms: (a) An optical image of an axial mouse brain tissue section after nano-DESI MSI analysis. Ion images of hemoglobin proteins proteoforms normalized to TIC: (b) \(m/z\) 948.121116\(^+\), 15,154 Da, RCG34342, isoform CRA\_a, (c) \(m/z\) 893.8187 \(^+\), 15,178 Da, hemoglobin subunit alpha (HBA1), (d) \(m/z\) 894.9384 \(^+\), 15,197 Da, hemoglobin subunit alpha (HBA1), (e) \(m/z\) 896.0538 \(^+\), 15,216 Da, hemoglobin subunit alpha (HBA1), (f) \(m/z\) 952.4971 \(^+\), 15,224 Da, hemoglobin subunit alpha (HBA1), (g) \(m/z\) 953.6171 \(^+\), 15,242Da, hemoglobin subunit alpha (HBA1), (h) \(m/z\) 954.6166 \(^+\), 15,258 Da, hemoglobin subunit alpha (HBA1), (i) \(m/z\) 992.2816\(^+\), 15,861 Da, hemoglobin subunit beta-1 (HBB-1), (j) \(m/z\) 993.5293 \(^+\), 15,881 Da, (k) \(m/z\) 936.6135 \(^+\), 15,905Da, (l) \(m/z\) 898.3611 \(^+\), 16,152 Da, (m) \(m/z\) 899.5815 \(^+\), 16,174 Da, (n) \(m/z\) 900.6377 \(^+\), 16,193 Da. Scale bar: 3mm.
Figure S11. Hemoglobin proteoforms: (a) An optical image of an axial mouse brain tissue section before nano-DESI MSI analysis. Ion images of hemoglobin proteins proteoforms normalized to TIC: (b) m/z 948.121116+, 15,154 Da, RCG34342, isoform CRA_a, (c) m/z 893.818717+, 15,178 Da, hemoglobin subunit alpha (HBA1), (d) m/z 950.870916+, 15,197 Da, hemoglobin subunit alpha (HBA1), (e) m/z 896.053817+, 15,216 Da, hemoglobin subunit alpha (HBA1), (f) m/z 952.497116+, 15,224 Da, hemoglobin subunit alpha (HBA1), (g) m/z 953.741616+, 15,243 Da, hemoglobin subunit alpha (HBA1), (h) m/z 954.616615+, 15,259 Da, hemoglobin subunit alpha (HBA1), (i) m/z 992.345316+, 15,861 Da, hemoglobin subunit beta-1 (HBB-1), (j) m/z 1059.831415+, 15,882 Da (k) m/z 936.613517+, 15,905Da, (l) m/z 1077.829115+, 16,152 Da, (m) m/z 1080.695615+, 16,193 Da. Scale bar: 3mm.
Figure S12. Hemoglobin proteoforms: (a) An optical image of an axial mouse brain tissue section before nano-DESI MSI analysis. Ion images of hemoglobin proteins proteoforms normalized to TIC: (b) m/z 948.121816+, 15,154 Da, RCG34342, isoform CRA_a, (c) m/z 949.617316+, 15,178 Da, hemoglobin subunit alpha (HBA1), (d) m/z 894.938317+, 15,197 Da, hemoglobin subunit alpha (HBA1), (e) m/z 951.994516+, 15,216 Da, hemoglobin subunit alpha (HBA1), (f) m/z 952.493516+, 15,224 Da, hemoglobin subunit alpha (HBA1), (g) m/z 953.617116+, 15,242 Da, hemoglobin subunit alpha (HBA1), (h) m/z 992.345316+, 15,861 Da, hemoglobin subunit beta-1 (HBB-1), (j) m/z 993.527916+, 15,881 Da, (k) m/z 936.613117+, 15,905 Da, (l) m/z 1077.829115+, 16,152 Da, (m) m/z 900.637718+, 16,193 Da. Scale bar: 3mm.
Figure S13. MS/MS spectrum of 784.567+, 5,485 Da, the unmodified cytochrome c oxidase subunit 7c, mitochondrial (COX7c) and its fragmentation map at 30 NCE. Isolation window: 3 m/z.

**Sequence coverage: 57%.**
Figure S14. MS/MS spectrum of 998.7910+, 9.978 Da, cytochrome c oxidase subunit 6B1 (COX6B1), N-terminal acetylation and its fragmentation map at 25 NCE. Isolation window: 3 m/z.

Sequence coverage: 15%.
Figure S15. MS/MS spectrum of 952.639+, 8,565 Da, the unmodified polyubiquitin and its fragmentation map at 25 NCE. Isolation window: 3 m/z.

Sequence coverage: 49%.
**Figure S16.** MS/MS spectrum of 709.93⁺, 4,963 Da, thymosin β-4, acetylation and its fragmentation map at 25 NCE. Isolation window: 3 m/z.

N-terminal acetylation

K3 acetylation

Sequence coverage: 55%.
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**Figure S17.** MS/MS spectrum of 679.21$^+$, 4,747 Da, thymosin β-4, acetylation and its fragmentation map at 25 NCE. Isolation window: 3 m/z.

Sequence coverage: 23%.

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**Figure S17.** MS/MS spectrum of 679.21$^+$, 4,747 Da, thymosin β-4, acetylation and its fragmentation map at 25 NCE. Isolation window: 3 m/z.
Figure S18. MS/MS spectrum of 812.52 Da, 8,927 Da, the unmodified ATP synthase-coupling factor 6, mitochondrial and its fragmentation map at 25 NCE. Isolation window: 3 m/z.

Sequence coverage: 15%.
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Figure S19. MS/MS spectrum of 813.25^{10+}, 8,122 Da, the unmodified ATP synthase subunit e, mitochondrial and its fragmentation map at 25 NCE. Isolation window: 3 m/z.

Sequence coverage: 13%.
**Figure S20.** MS/MS spectrum of 765.40^{13+}, 9,937 Da, acyl-CoA binding protein, acetylation and its fragmentation map at 25 NCE. Isolation window: 3 m/z.

**N-terminal acetylation**

**K3 acetylation**

Sequence coverage: 45%.
**Figure S21.** MS/MS spectrum of 741.8019+, 14,075 Da, the unmodified myelin basic protein and its fragmentation map at 25 NCE. Isolation window: 0.5 m/z.
**Figure S22.** MS/MS spectrum of 744.2319+, 14,121 Da, myelin basic protein, N-terminal acetylation and its fragmentation map at 25 NCE. Isolation window: 0.5 m/z.

Sequence coverage: 24%.
**Figure S23.** MS/MS spectrum of 745.02$^{19+}$, 14,136 Da, myelin basic protein, N-terminal acetylation, methionine sulfoxide and its fragmentation map at 25 NCE. Isolation window: 0.5 m/z.

Sequence coverage: 14.3%.
**Figure S24.** MS/MS spectrum of 745.8119+, 14,151 Da, myelin basic protein, phosphorylation and its fragmentation map at 25 NCE. Isolation window: 0.5 m/z.
Figure S25. MS/MS spectrum of 747.7119+, 14,187 Da, myelin basic protein, di-methylation, phosphorylation and its fragmentation map at 25 NCE. Isolation window: 0.5 m/z. Note that database search did not report a match for 14,187 Da MBP. This proteoform was manually annotated by matching its intact mass with all possible combinations of PTMs reported from Swiss-Prot. MS/MS data was used to confirm the assignment. This proteoform was assigned to level 2A due to unlocalized PTMs. The above fragmentation map represents this proteoform with the best sequence coverage matched to MS/MS data.
Figure S26. MS/MS spectrum of 748.3919+, 14,200 Da, myelin basic protein, N-terminal acetylation, phosphorylation and its fragmentation map at 25 NCE. Isolation window: 0.5 m/z.

**N-terminal acetylation, S118 phosphorylation**

Sequence coverage: 13%.
Figure S27. MS/MS spectrum of 752.49$^{19+}$, 14,278 Da, myelin basic protein, N-terminal acetylation, diphosphorylation and its fragmentation map at 25 NCE. Isolation window: 0.5 m/z.

Sequence coverage: 6%.
**Figure S28.** MS/MS spectrum of 682.3527+, 18,397 Da, myelin basic protein, N-terminal acetylation and its fragmentation map at 25 NCE. Isolation window: 0.5 m/z.
**Figure S29.** MS/MS spectrum of 948.1216+, 15,154 Da, RCG34342, isoform CRA_a, and its fragmentation map at 23 NCE. Isolation window: 3 m/z.

Sequence coverage: 31%.
Figure S30. MS/MS spectrum of 893.8117+, 15,178 Da, hemoglobin subunit alpha (HBA1), phosphorylation and its fragmentation map at 23 NCE. Isolation window: 3 m/z.
**Figure S31.** MS/MS spectrum of 952.4816\(^{18}\), 15,224 Da, hemoglobin subunit alpha (HBA1), S44N and its fragmentation map at 25 NCE. Isolation window: 0.5 \(m/z\).

Sequence coverage: 16%.
Figure S32. MS/MS spectrum of 953.6216+, 15,242 Da, hemoglobin subunit alpha (HBA1), N-terminal acetylation and its fragmentation map at 23 NCE. Isolation window: 3 m/z.

Sequence coverage: 19%.
Figure S33. MS/MS spectrum of $954.61^{16+}$, 15,258 Da, hemoglobin subunit alpha (HBA1), N6-succinyl-L-lysine and its fragmentation map at 25 NCE. Isolation window: 0.5 m/z.

Sequence coverage: 6%.
**Figure S34.** MS/MS spectrum of 992.28$^{16+}$, 15,861 Da, hemoglobin subunit beta-1, R104 di-methylation and its fragmentation map at 23 NCE. Isolation window: 3 m/z.

Sequence coverage: 20%.
Figure S35. MS/MS spectrum of 1027.12\textsuperscript{10+}, 10,261 Da, dynein lightchain 2, cytoplasmic, acetylation and its fragmentation map at 30 NCE. Isolation window: 3 m/z.

Sequence coverage: 23\%.
Figure S36. MS/MS spectrum of 627.249^+, 5,636 Da, the unmodified ATP synthase subunit epsilon, mitochondrial and its fragmentation map at 23 NCE. Isolation window: 3 m/z.

Figure S37. MS/MS spectrum of 742.6619^+, 14,091 Da, myelin basic protein, PTM uncharacterized at 25 NCE. Isolation window: 0.5 m/z.
Figure S38. MS/MS spectrum of 743.3819+, 14,105 Da, myelin basic protein, PTM uncharacterized at 25 NCE. Isolation window: 0.5 m/z.

Figure S39. MS/MS spectrum of 749.2919+, 14,217 Da, myelin basic protein, PTM uncharacterized at 25 NCE. Isolation window: 0.5 m/z.

Figure S40. MS/MS spectrum of 750.3919+, 14,238 Da, myelin basic protein, PTM uncharacterized at 25 NCE. Isolation window: 0.5 m/z.
**Figure S41.** MS/MS spectrum of 682.3627+, 18,412 Da, myelin basic protein, PTM uncharacterized at 25 NCE. Isolation window: 0.5 m/z.

**Figure S42.** MS/MS spectrum of 993.61^{16+}, 15,881 Da, hemoglobin subunit beta-1 or beta-2, PTM uncharacterized at 25 NCE. Isolation window: 0.5 m/z.

**Figure S43.** MS/MS spectrum of 936.62^{27+}, 15,905 Da, hemoglobin subunit beta-1 or beta-2, PTM uncharacterized at 25 NCE. Isolation window: 0.5 m/z.
Figure S44. MS/MS spectrum of 1077.89^{15+}, 16,152 Da, hemoglobin subunit beta-1 or beta-2, PTM uncharacterized at 25 NCE. Isolation window: 0.5 m/z.

Figure S45. MS/MS spectrum of 899.58^{18+}, 16,174 Da, hemoglobin subunit beta-1 or beta-2, PTM uncharacterized at 25 NCE. Isolation window: 0.5 m/z.

Figure S46. MS/MS spectrum of 900.64^{18+}, 16,193 Da, hemoglobin subunit beta-1 or beta-2, PTM uncharacterized at 25 NCE. Isolation window: 0.5 m/z.
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