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TITLE:

Laser Microdissection-Based Protocol for the LC-MS/MS Analysis of the Proteomic Profile of Neuromelanin Granules

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KEYWORDS:

Neuromelanin granules, laser microdissection, mass spectrometry, *substantia nigra pars compacta*, parallel reaction monitoring, data-dependent acquisition

SUMMARY:

A robust protocol is presented here for isolating neuromelanin granules from human *post-mortem substantia nigra pars compacta* tissue *via* laser microdissection. This revised and

optimized protocol massively minimizes the required time for sample collection, reduces the required sample amount, and enhances the identification and quantification of proteins by LC-MS/MS analysis.

ABSTRACT:

Neuromelanin is a black-brownish pigment, present in so-called neuromelanin granules (NMGs) in dopaminergic neurons of the *substantia nigra pars compacta*. Besides neuromelanin, NMGs contain a variety of proteins, lipids, and metals. Although NMGs-containing dopaminergic neurons are preferentially lost in neurodegenerative diseases like Parkinson's disease and dementia with Lewy bodies, only little is known about the mechanism of NMG formation and the role of NMGs in health and disease. Thus, further research on the molecular characterization of NMGs is essential. Unfortunately, standard protocols for the isolation of proteins are based on density gradient ultracentrifugation and therefore require high amounts of human tissue. Thus, automated laser microdissection (LMD)-based protocol is established here which allows the collection of NMGs and surrounding *substantia nigra* (SN) tissue using minimal amounts of tissue in an unbiased, automatized way. Excised samples are subsequently analyzed by mass spectrometry to decipher their proteomic composition. With this workflow, 2,079 proteins were identified of which 514 proteins were exclusively identified in NMGs and 181 in SN. The present results have been compared with a previous study using a similar LMD-based approach reaching an overlap of 87.6% for both proteomes, verifying the applicability of the revised and optimized protocol presented here. To validate current findings, proteins of interest were analyzed by targeted mass spectrometry, e.g., parallel reaction monitoring (PRM)-experiments.

INTRODUCTION:

Every tissue consists of a heterogeneous mixture of different cell types, but the specific isolation of one cell type often is indispensable for a more precise characterization. Laser microdissection (LMD), coupling a microscope with a laser application, is a powerful tool for the specific isolation of tissue areas, single cells, or cellular substructures out of a complex composite. The application of LMD in combination with mass spectrometry (LMD-MS) has already been successfully implemented for several research questions, including isolation of DNA[1], RNA[2], and proteins[3–5]. In this protocol, a revised and optimized LMD-MS protocol is described for the proteomic analysis of human *post-mortem* brain tissue and sub-cellular components to decipher novel pathomechanisms of Parkinson's disease.

Neuromelanin is a black, nearly-insoluble pigment found in the catecholaminergic, dopamine-producing neurons of the *substantia nigra pars compacta*[6]. Together with proteins and lipids, it accumulates in organelle-like granules surrounded by a double membrane, called neuromelanin granules (NMGs)[7–9]. NMGs can be observed from the age of three years in humans increasing in quantity and density during the aging process[10,11]. To date, there is no definite hypothesis on neuromelanin formation, but one assumption is that neuromelanin is formed through the oxidation of dopamine[12]. Other hypotheses are based on enzymatic production of neuromelanin (e.g., tyrosinase)[13]. Neuromelanin itself was found to have a high binding affinity to lipids, toxins, metal ions, and pesticides. Based on these findings, the formation of NMGs is assumed to protect the cell from the accumulation of toxic and oxidative substances and from environmental

toxins[14,15]. Besides this neuroprotective function, there is evidence that neuromelanin may cause neurodegenerative effects, e.g., by iron saturation and the subsequent catalysis of free radicals[16,17]. Furthermore, neuromelanin released during neurodegenerative processes can be decomposed by hydrogen peroxide, which could accelerate necrosis by reactive metals and other toxic compounds previously bound to neuromelanin and may contribute to neuroinflammation and cellular damage[18]. However, until now the exact role of NMGs in neurodegenerative processes like in the course of Parkinson's disease is not clearly understood. Still, NMGs seem to be involved in the pathogenesis of Parkinson's disease and their specific analysis is of utmost importance to unravel their role in neurodegeneration. Unfortunately, common laboratory animals (e.g., mice and rats) and cell lines lack NMGs[19]. Therefore, researchers especially rely on *post-mortem* brain tissue for their analysis. In the past, NMG isolation by density gradient centrifugation relied on the availability of high amounts of *substantia nigra* tissue[20,21]. Today, LMD presents a versatile tool to specifically isolate NMGs from human brain samples to then analyze them by LC-MS/MS.

In this protocol, an improved and automated version of a previous protocol[22] is presented for the isolation of NMGs and surrounding tissue (SN), enabling a faster sample generation, higher numbers of identified and quantified proteins, and a severe reduction of required tissue amounts.

PROTOCOL:

The use of human brain tissue was approved by the ethics committee of the Ruhr-University Bochum, Germany (file number 4760-13), according to German regulations and guidelines. This protocol has been applied on commercially obtained *substantia nigra pars compacta* tissue slices. A graphical overview of the presented protocol is shown in **Figure 1**.

1. Tissue sectioning

1.1. Precool the cryostat chamber.

NOTE: Every tissue requires different cryostat temperatures, which can be found in the respective vendor protocol.

1.2. Clean the stainless-steel knife with 70% ethanol and install it into the blade holder.

1.3. Transfer the tissue from the -80 °C freezer to the cryostat using an icebox and let it adjust to the cryostat chamber temperature for 15 min.

1.4. Unambiguously label membrane slides using a pencil.

NOTE: PET/PEN membrane slides are required for the LMD-based sample collection. Handle the PET/PEN membrane slides with care as they are extremely fragile.

1.5. Apply a drop of commercial frozen section medium on the tissue holder. Before it is

completely frozen, place the tissue onto the frozen section medium and let it harden, so that the tissue is connected with the tissue holder.

1.6. Install the tissue holder in the cryostat chamber and adjust its orientation before starting sectioning. Optimal holder orientation depends on the orientation of the tissue.

NOTE: It may be necessary to trim the tissue until the section plane needed for the slices is reached.

1.7. Before the tissue area of interest is reached, adjust the cutting setting to the desired tissue thickness.

NOTE: 5 or 10 µm is the suggested thickness for this protocol as 20 µm thick sections were found to be incompatible with the LMD-based sample collection[22].

1.8. Cut two sections and discard them.

1.9. Put down the Anti-roll Plate.

1.10. Cut a section of the tissue, open the Anti-roll Plate carefully, take a membrane slide, and prevent tissue folding while placing the tissue section on the membrane slide.

NOTE: Storing the membrane slides at room temperature prior to adhesion enables accurate sample attachment. Several sections may be placed on the same membrane slide but the tissue overlapping must be prevented.

1.11. Store tissue sections placed on membrane slides in the cryostat until sectioning is completed.

1.12. Store the cryosected tissue at -20 °C until further processing or directly proceed with the below procedure. Store the sectioned tissue slides at -80 °C until further use.

2. Laser Microdissection and Pressure Catapulting

NOTE: As neuromelanin granules are visible without any staining due to their black-brownish color, no staining is necessary for this protocol. Nevertheless, different staining procedures can be combined with this protocol if required. Keep in mind that the use of blocking solutions or antibodies will influence the LC-MS/MS analyses.

2.1. Switch on the MicroBeam system and open the associated software on the computer (see **Table of Materials**).

2.2. Place the tissue membrane slide in the SlideHolder on the RoboStage with the tissue facing upwards.

NOTE: Depending on the LMD device, it may be necessary to place the membrane slide such that the tissue is facing down. In general, sample collection is performed in a temperature-controlled environment to ensure optimal and reproducible conditions.

2.3. Set the microscope to the desired magnification (50-fold is used here) for the overview scans.

2.4. Use the Scan function, which can be found in the Navigator window of the software interface, to acquire an overview scan of the tissue section. Search for the top-left corner and the bottom-right corner of the area of interest and select them in the software interface. Then, select **Scan all ROIs** to perform the scans.

NOTE: Overview scans are not mandatory, but they enable better orientation in the slide and can be saved for later usage.

2.5. Adjust the magnification of the microscope for the appropriate tissue, which is 400-fold in the present case of neuromelanin granules.

2.6. Search for an area with neuromelanin granules. Select **Field of View Analysis** in the software interface, select **Invert Result,** and set the threshold for the RGB channels so that only neuromelanin granules are highlighted in red in the preview window. Click on **OK** to use the adjusted settings for the field of view.

NOTE: It may occur that smaller objects having a dark color also get selected. To account for that, discard all objects covering an area smaller than 100 μm^2 before isolating neuromelanin granules. To do this, open the Element List by clicking on the icon in the toolbar, select the slide under consideration and order elements by area. Select those with areas smaller than 100 μm^2 and delete them.

2.7. Adjust laser settings using an area of the slide that is covered by the membrane only.

NOTE: It is suggested to use the **Cut Laser Adjustment Wizard** and follow the instructions of the software. Required laser settings may differ between different slides. For 5 µm sections with 400-fold magnification, typical settings are 32 energy and 51 focus for cutting, and 28 energy and -1 focus for laser pulse catapulting (LPC).

2.8. Adjust speed settings for **positioning** and **cutting** to ensure proper isolation.

NOTE: 30% speed was found to be optimal for NMG isolation.

2.9. Fill the sample collection tube cap with 50 µL ultrapure water and insert the cap into the collector of the RoboMover.

NOTE: The tube collector used for present experiments can carry one sample collection tube at

a time.

2.10. Position the RoboMover above the RoboStage II using the software interface to start sample collection.

NOTE: To do this, open the RoboMover window, which displays the collector. Click on the sample collection tube cap displayed in the RoboMover window to move the cap to the working area. Adjust the optimal moving and working height in the RoboMover window. Otherwise, the water in the cap may drop onto the slide or the catapulted objects will not reach the cap.

2.11. Start the laser. Control energy and focus settings during the laser process and adjust the settings if necessary. Ensure proper isolation and catapulting of the isolated objects into the sample collection tube cap for at least the first ten objects.

NOTE: Proper isolation and catapulting have to be checked visually. Both should result in a tissue-free area of the size of the pre-selected object in the tissue slice (see **Figure 2C,D**). Adjust the laser settings if the object stays attached to the tissue slice after cutting and catapulting. For catapulting, the CenterRoboLPC option is found to be well suited for NMG isolation. The catapulting settings can be adjusted for each selected object in the Element List.

2.12. When sampling is completed, navigate the RoboMover to its starting position. Remove the sample collection tube.

NOTE: When the number of collected objects is rather low and objects are big enough, sample collection can be ensured by clicking on **Cap Check**, which will place the sample collection tube cap under the microscope so that the number of objects inside of the water in the cap can be counted (see **Figure 2H**).

2.13. Spin down the sample using a centrifuge. Short spins of 5 s with increasing centrifugal force due to acceleration of the centrifuge were found to be sufficient. At this point, store samples at -80 °C, as all samples should be further processed together.

NOTE: For the comparison of the proteomic profile, the tissue surrounding the NMGs was also isolated after their excision. The isolation of the surrounding tissue was performed at 50-fold magnification.

2.14. Dry the samples in a vacuum concentrator. 1.5 h were found to be sufficient for 50 µL of water.

2.15. Solubilize and lyse the tissue in 40 µL formic acid for 20 min (room temperature).

2.16. Enhance tissue lysis by sonication at 45 kHz (kilohertz) for 5 min in a sonication bath. Fill the sonication bath with ice to prevent tubes from melting. Store the samples at -80 °C until further processing.

3. Tryptic digestion

3.1. Defreeze samples on ice.

3.2. Completely dry the samples in a vacuum concentrator.

3.3. Fill up the sample with 50 µL of a suitable digestion buffer, e.g., 50 mM ammonium bicarbonate.

3.4. After the addition of 1.25 µL of 200 mM 1,4-dithiothreitol, incubate the samples for 30 min at 60 °C and 300 rpm using a thermomixer and cool them down to room temperature (RT) afterwards.

3.5. Then, incubate samples at RT for 30 min in the dark after the addition of 1.36 µL 0.55 M iodoacetamide.

3.6. Add a suitable amount of trypsin to the samples and incubate the samples overnight (~16 h) at 37 °C.

NOTE: For 1,000,000 µm², 0.1 µg of trypsin was found to be sufficient.

3.7. Add 2.6 µL of 10% trifluoroacetic acid (TFA) to the samples to stop the digestion (end concentration of 0.5% TFA).

3.8. Completely dry the sample using a vacuum concentrator. Then, fill samples up to a defined final volume with 0.1% TFA. NMG samples were filled up to 20 µL of which 5 µL were used for one mass spectrometric (MS) experiment.

3.9. Store the samples at -80 °C until further usage. Determine peptide concentration by amino acid analysis or another suitable quantification method (e.g., Direct Detect).

NOTE: Low sample amounts may not be quantifiable using the mentioned techniques. To ensure identical sample loading, each sample should contain the same amount of isolated tissue and every sample should be treated equally.

4. High-performance liquid chromatography and mass spectrometry

NOTE: The following high-performance liquid chromatography (HPLC) mass spectrometric (MS) analysis are optimized for the specific LC system with a trapping column device and mass spectrometer used here (see **Table of Materials**). For other LC and MS systems, adaption of parameters is recommended.

4.1. Using the software Xcalibur, adjust the HPLC settings as follows.

4.1.1. Trap column: Set temperature to 60 °C, flow rate to 30 µL/min, running buffer to 0.1% trifluoroacetic acid.

4.1.2. Analytical C18 reversed-phase column: Set temperature to 60 °C, flow rate to 30 µL/min, running buffer A to 0.1% trifluoroacetic acid, running buffer B to 84% acetonitrile, and gradient to 5%–30% running buffer B over 98 min.

NOTE: Adaption of the gradient may be inevitable and is strongly recommended when using different tissues or cells. Total gradient time may vary due to sample loading at the beginning of the gradient and sample washing at the end of the gradient. The total gradient in this protocol consists of 7 min sample loading and additional column wash for 15 min resulting in a total gradient time of 120 min.

4.2. Create a data-dependent acquisition (DDA) method using the XCalibur Instrument Setup, which can be found in the HPLC software roadmap menu.

4.3. In the **Global Parameters** tab, define the infusion mode **Liquid Chromatography**, the **Expected LC Peak Width** (30 s), and the **Default charge state** (2).

4.4. Proceed to the **Scan Parameters** tab and add the following scans and filters in the order mentioned: **MS OT**, **MIPS**, **Intensity**, **Charge State**, **Dynamic exclusion**, and **ddMS2 OT HCD**.

NOTE: The detailed parameter settings for each scan and filter can be found in **Supplementary Table 1**. Optimal MS and DDA settings might vary for the specific mass spectrometer used as well as the sample type and should be, therefore, adapted.

4.5. Prepare samples by dissolving 200-400 ng of sample peptides in a defined volume of 0.1% TFA in inert mass spectrometric glass vial inlets. If concentration determination is not applicable due to low sample amount, verify identical sample loading by comparing the Total Ion Current (TIC).

NOTE: To do this, open the resulting file of mass spectrometric measurement in a suitable software, e.g., FreeStyle, and check the chromatogram. Intensities should be comparable for all samples. A representative TIC is shown in **Figure 3**.

4.6. Analyze the raw data obtained using a proteomic suitable software, e.g., MaxQuant[23], Progenesis QI for Proteomics, or Proteome Discoverer, and perform a statistical data analysis based on the research question.

5. Analysis of proteomic raw data using MaxQuant

NOTE: A detailed information on MaxQuant parameters is provided in **Supplementary Table 2**. They are briefly described below.

5.1. Load raw files into the MaxQuant software in the raw data header by clicking **Load**.

5.2. Assign sample names by clicking on **Set Experiment**.

5.3. Define group-specific parameters. First, add modifications. Due to sample processing, choose **Deamidation (NQ)**, **Oxidation (M)**, and **Carbamidomethylation (N-term)** as variable modifications, and add **Carbamidomethylation (C)** as fixed modification.

5.4. Choose **Trypsin** as a digestion enzyme in the **Digestion** tab.

5.5. Add the label free quantification option **LFQ** in the **Label Free Quantification** tab. If more than 10 files are to be processed, choose the **Fast LFQ** option to shorten the processing time. Add **iBAQ** option as a measure for protein quantification[24].

5.6. Ensure that all other group-specific parameters remain in factory settings.

5.7. Proceed to the **Global Parameters** tab and add the FASTA file derived from uniprot.org in the **Sequences** tab. Modify the identifier rule accordingly and add the taxonomy ID, in this case, 9606 for *homo sapiens*.

5.8. For protein quantification choose **Unique and Razor** peptides.

5.9. Ensure that all other global parameters remain in factory settings.

5.10. Click on Start and retrieve the **proteingroups.txt** output after MaxQuant analysis for further analysis in Perseus.

6. Statistical analysis using Perseus

6.1. Load the **proteingroups.txt** file in Perseus, add the iBAQ values as main columns, and sort all other columns according to their type.

6.2. Filter out decoys and contaminants by filtering rows based on the categorical column.

6.3. Filter results based on valid values. In the present case, with only two samples included in

the analysis, a minimum number of one valid value was chosen.

6.4. Export the Perseus output in .txt format for further processing, for example, in Excel, and evaluate the results regarding the research question.

7. Validation of selected proteins

NOTE: Commonly used methods for validation of MS data are, for example, immunological staining or western blot. Due to the dark color and the autofluorescence of neuromelanin, immunological staining of proteins inside of neuromelanin granules either with horseradish peroxidase- or fluorophore-conjugated antibodies are not applicable. For Western Blot analysis, very large amounts of *post-mortem* tissue would be necessary. Therefore, selected proteins are validated by targeted mass spectrometry, and in the present case, parallel reaction monitoring (PRM)-experiments were set up.

7.1. Select proteins for validation. Choose peptides of these proteins already detected in DDA experiments. Peptides should contain no missed cleavages or modifications to ensure a reliable quantification.

NOTE: There can be several reasons for the validation of one specific protein, for example, differential abundances in the investigated conditions. For the representative results, cytoplasmic dynein 1 heavy chain 1 has been selected, which was found to be equivalently abundant in NMG and SN samples and could therefore be used as a reference to ensure equal sample loading.

7.2. Use the selected peptides to set up the first version of a PRM-method using the HPLC software. Keep all chromatography and global parameters settings from the DDA method.

7.3. Add **MS OT** and **tMS^2 OT HCD** as scan types. Ensure that the settings for **MS OT** are the same as for the DDA method. Detailed settings for the PRM method can be found in **Supplementary Table 1.**

7.4. For **tMS^2 OT HCD**, add selected peptides as an inclusion list. Therefore, add the amino acid sequence and the m/z value observed in the DDA measurements. For the first PRM experiment, do not add retention time windows or set **t start** to 0 and **t stop** to 120 (for a 120-min gradient).

7.5. Evaluate the PRM method after the measurement using suitable software, for example, Skyline, and obtain the retention time of the peptides added to the inclusion list. For included peptides, check that comparable peaks are observable for at least three precursor ions in MS1 scans and five fragment ions in MS2 scans with low mass error (±5 ppm).

7.6. Refine the PRM method, for example, by increasing the resolution for the **tMS^2 OT HCD** scan and adding retention time windows to the inclusion list.

NOTE: Retention time windows of 3 min were found to be well-suited in present experiments (observed retention time in first PRM experiment ± 1.5 min).

7.7. With the refined PRM-method, perform quantification of peptides and proteins of interest based on the peak area both on MS1 and MS2 levels with suitable software.

REPRESENTATIVE RESULTS:

The specific isolation of NMGs and SN tissue is the most important step for the successful application of this protocol. Using the **Field of View Analysis** function in the vendor-provided software of the LMD, NMGs can be automatically selected in a color-dependent manner. Therefore, tissue areas containing NMGs (**Figure 2A**) have to be identified and a Field of View Analysis with adjusted color thresholds has to be performed, resulting in the labeling of NMGs (**Figure 2B**). After filtering of objects covering an area below 100 µm², only NMGs should remain labeled for isolation (**Figure 2C**). Precise isolation of the labeled NMGs is achieved after laser settings were adjusted (**Figure 2D**). After the isolation of NMGs (**Figure 2E**), SN tissue can be selected with 50-fold magnification (**Figure 2F**) and isolated (**Figure 2G**) for comparison of the proteomic profile. For SN tissue, isolated objects can be visualized using the **Cap Check** function (**Figure 2H**). For both sample types, NMG and SN, isolation of 500,000 µm² of brain tissue was found to be sufficient for this protocol, enabling a minimum of three MS runs per sample.

A representative example of a 120 min DDA experiment is shown in **Figure 3** (as the main column is washed in the last 15 min of the measurement, the chromatogram is cropped just before 105 min). The applied method should allow a sample elution over the complete gradient, creating sharp and concise peaks and the intensity of the Total Ion current (TIC) should be comparable across all samples.

Application of the presented protocol on one sample of 500,000 µm² of NMG and one sample of 1,000,000 µm² SN tissue with adjusted volumes for MS samples (5 µL for NMG and 2.5 µL for SN) to ensure identical peptide load, resulted in the identification of 1,898 protein groups (PGs) in the NMG sample and 1,565 PGs in the SN sample. Further comparison revealed 1,384 PGs to be identified in both samples, while 514 PGs were exclusively identified in NMG and 181 PGs in SN tissue (**Figure 4**). In total, 2,079 PGs were identified in this representative experiment. Comparison with a reference dataset from a former study[22] showed that 87.6% of the PGs reported in that study could also be identified by the present revised and automated protocol, proving its applicability. Furthermore, the number of identified PGs could be improved by 1,143.

As minimal sample amounts do not allow the application of classic validation methods such as Western Blots, validation of proteins of interest can be achieved by targeted mass spectrometric approaches, e.g., PRM. Representative results for the peptide ESPEVLLTLDILK of the protein cytoplasmic dynein 1 heavy chain 1 are shown in **Figure 5**. The iBAQ value of this protein was found to be slightly higher in NMGs compared to SN in the DDA measurements, which could be verified by PRM-experiments based on the peak area on MS1- (**Figure 5A,B,E**) and MS2-level (**Figure 5C,D,F**).

FIGURE AND TABLE LEGENDS:

Figure 1: Workflow for the proteomic characterization of neuromelanin granules (NMGs) and surrounding tissue (SN). NMG and SN samples were isolated from tissue slices *via* laser microdissection (LMD). Proteins were isolated and tryptic in-solution digestion was performed. The resulting peptides were analyzed *via* LC-MS/MS measurements in data-dependent acquisition (DDA) mode. Data analysis was performed using MaxQuant and Perseus software. Validation of selected proteins was carried out with parallel reaction monitoring (PRM) experiments. PRM-data was analyzed using Skyline software.

Figure 2: Selection and LMD-based isolation of NMG and SN samples. At first, an area containing NMGs, visible without further staining at 400-fold magnification, is placed under the microscope (**A**). After performing a Field of View Analysis, NMGs and other dark areas are selected (**B**). Only NMGs remain selected after filtering (**C**) and are isolated after laser settings are adjusted (**D**). After all NMGs are isolated (**E**), SN tissue is selected with 50-fold magnification (**F**) and isolated (**G**). As objects isolated for SN samples are quite big, they can be observed in the sample collection cap using the **Cap Check** function (**H**).

Figure 3: Total Ion Current (TIC) of a 120 min DDA measurement. The chromatogram shows the relative abundance of the ions corresponding to the eluting peptides over the retention time range from 0 to ~105 min. As the main column is washed between 105^{th} and 120^{th} min, the chromatogram is cropped at the 105^{th} min. The intensity of the highest peak is 2.86×10^8. Numbers above peaks indicate the retention time and the most abundant ion of that specific peak (BP=base peak).

Figure 4: Venn Diagram showing the correspondence of protein groups (PGs) identified in NMGs and SN tissue. In total, 1,898 PGs were identified in NMGs and 1,565 PGs in SN tissue, of which 1,384 PGs were identified in both tissue areas. 514 PGs were exclusively identified in NMG tissue, while 181 PGs were exclusively identified in SN tissue. The diagram was created using the online tool Venny[25].

Figure 5: Results of PRM-experiments for the peptide ESPEVLLTLDILK (cytoplasmic dynein 1 heavy chain 1, ++). Chromatograms on MS1- (**A**,**B**) and MS2-level (**C**,**D**), as well as peak areas on MS1- (**E**) and MS2-level (**F**), are shown for an exemplary sample of NMGs and SN tissue. Different colors are used to denote different precursors (on MS1-level) or product ions (on MS2-level). Chromatograms are displayed after Savitzky-Golay Smoothing was performed. Intensities and peak areas were comparable on MS1- (**A**,**B**,**E**) and MS2-level (**C**,**D**,**F**).

Supplementary Table 1: Parameters of the mass spectrometry experiments.

Supplementary Table 2: Parameters of the MaxQuant analysis.

DISCUSSION:

LMD is a widely applicable technique for the isolation of specific tissue areas, single cells, or subcellular structures. In the revised and automated protocol presented here, this technique is

applied for the specific isolation of neuromelanin granules (NMGs) and NMG-surrounding tissue (SN). Until now, two different approaches for the isolation of NMGs out of human *post-mortem* brain tissue were published and widely used:

a) A discontinuous sucrose gradient consuming 1 g of *substantia nigra* tissue[20]. As human *post-mortem substantia nigra* tissue is rare and of high interest for several research questions, it is unfortunately quite challenging to set up a large cohort study if high amounts of tissue are required per patient. Therefore, this approach was further improved reducing the required tissue amount to 0.15 g for sufficient isolation of NMGs[26]. However, still, at least one-half of a complete *substantia nigra pars compacta* was required.

b) The excision of NMGs using LMD. In 2016, Plum et al established a new protocol based on the precise excision of NMGs *via* LMD. With this protocol, the required sample amount could be reduced to ten 10 µm tissue sections, resulting in an impressive reduction of the required sample from 150 mg to 16.6 mg[22].

The optimized and automated LMD-based protocol presented here requires even lower sample amounts as thinner (5 µm compared to 10 µm) and fewer tissue sections (maximum of 7 compared to 8) had to be used and requires less time for sample generation (4 h per sample compared to 1–2 days) through the use of automatized NMG detection. Thus, the required sample collection time was shortened massively and the number of identified PGs could be drastically enhanced by applying an optimized LC-MS method and state-of-the-art instrumentation. This protocol can easily be adapted to other research questions and tissues.

For the adaptation of the presented protocol concerning user-defined research questions, the following aspects are highlighted based on experience:

a) Isolation of comparable sample amounts: As the expected peptide yield of this protocol is rather low compared to, for instance, cell culture or tissue lysates, determination of peptide concentration may not be possible. Thus, it is crucial that equal amounts of tissue are isolated *via* LMD, which can be estimated based on the tissue area of the selected objects. In the current setup, tissue areas of 500,000 µm² are sufficient for the generation of peptides for at least three MS measurements.

b) Trypsin-digestion: The duration of digestion and the trypsin concentration should be comparable across samples.

c) Adaption of parameters for different tissues: Depending on the tissue to analyze, the collected tissue amount needs to be adjusted thereby making it necessary to adjust the amount of added trypsin as well. The trypsin to protein ratio should not be lower than 1:40.

d) Limitation of the LMD process: For the LMD-based isolation of objects of interest, there are limitations when it comes to the size of selected objects and the thickness of slices. Due to tissue loss during the laser-based cutting of the tissue, objects smaller than 100 µm² were considered too small for isolation.

e) Adaption of LC and MS parameters: Depending on the LC and MS systems used, the amount of isolated tissue has to be increased (e.g., when operating with a microflow system) and MS parameters have to be adapted (e.g., when working with an ion-trap-based detector system).

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors declare no conflicts of interest.

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Figure 1

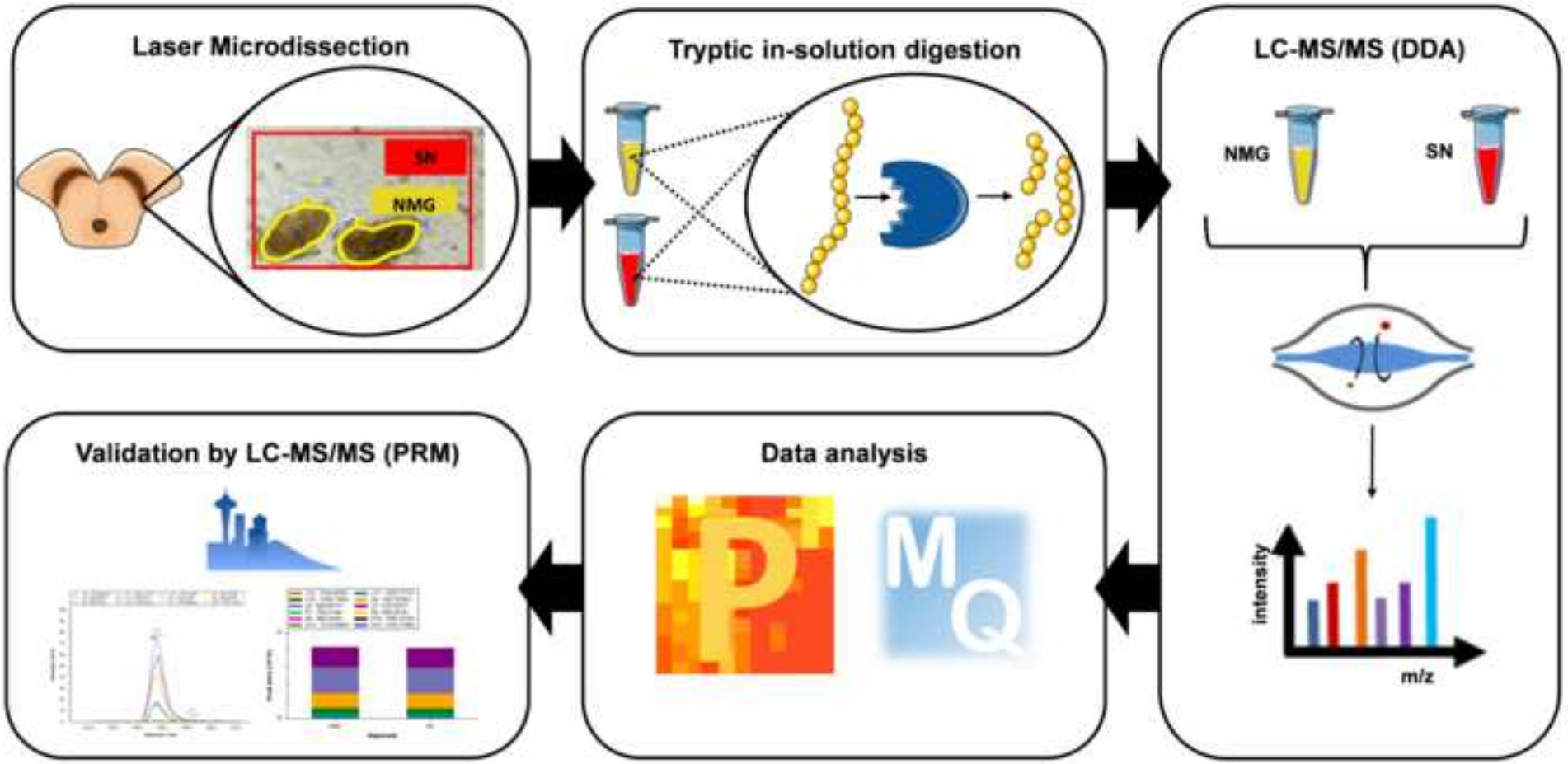


Figure 2

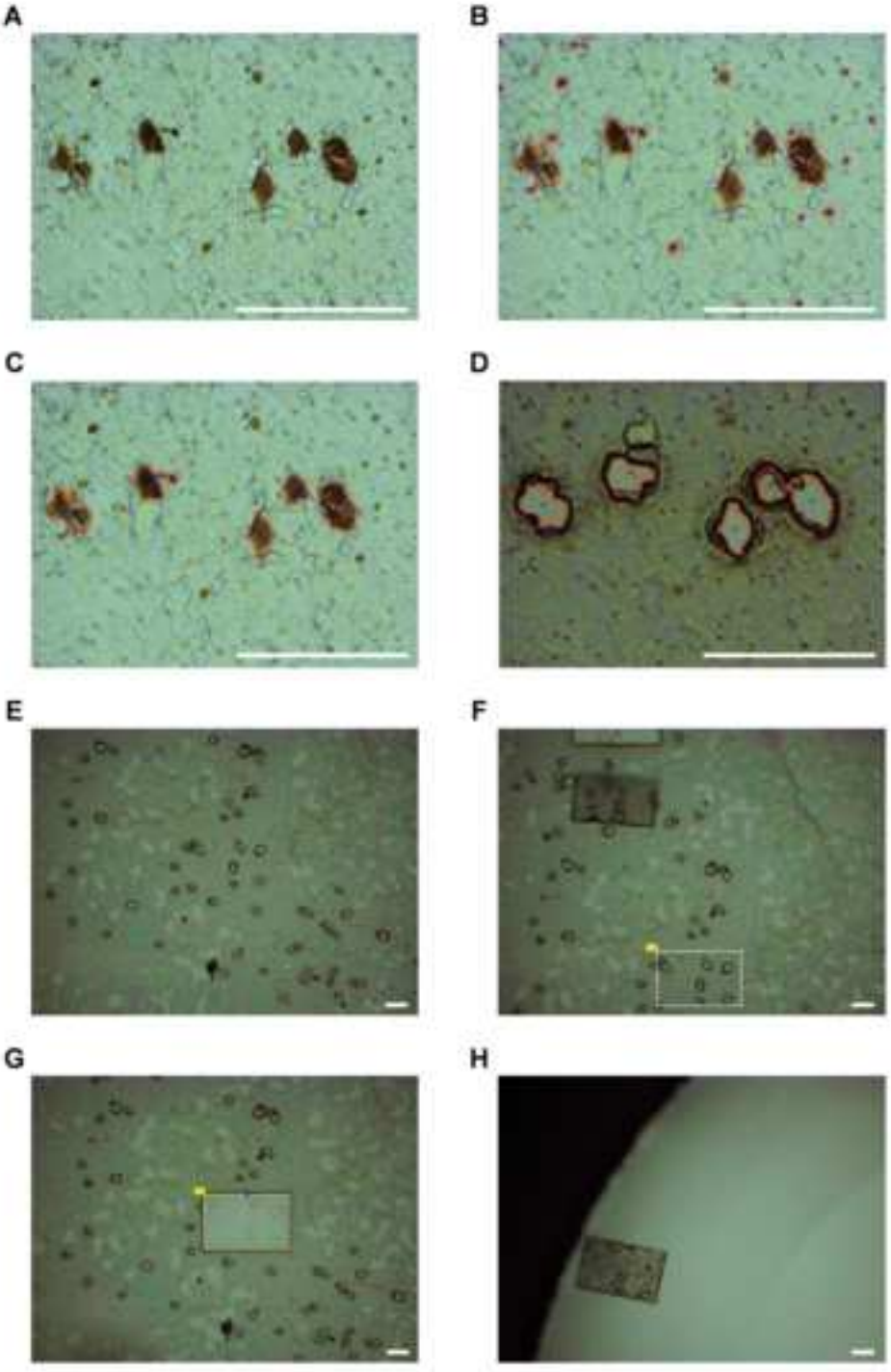


Figure 3

Click here to access/download;Figure;Figure3.tif

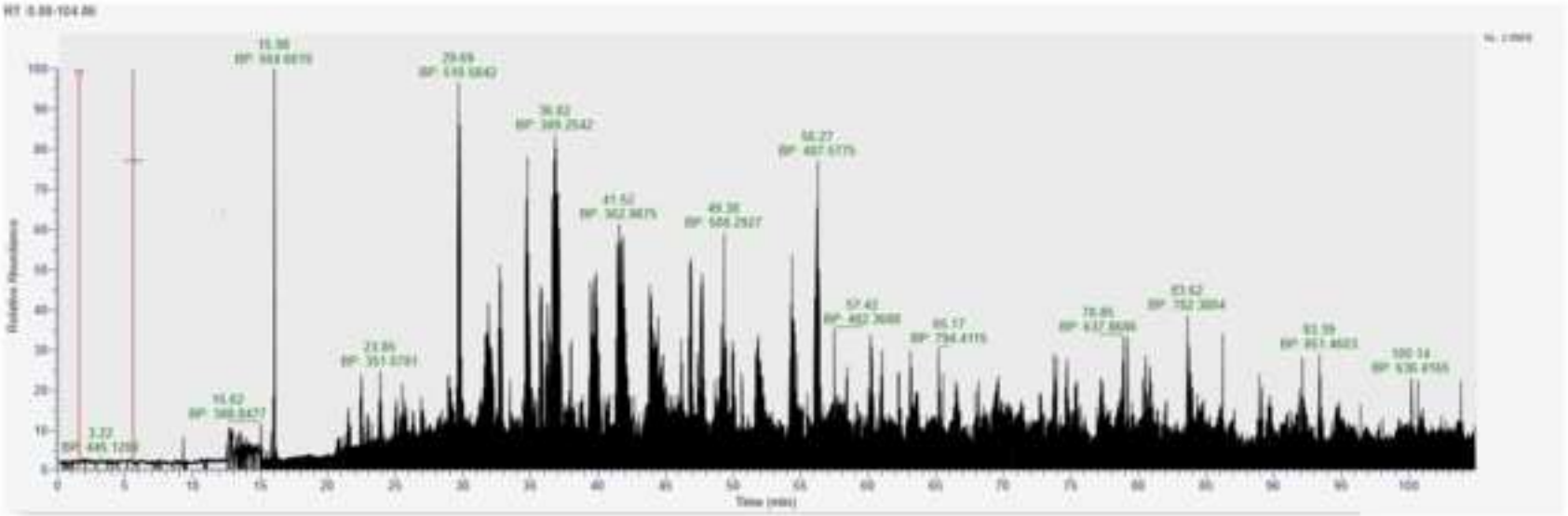


Figure 4

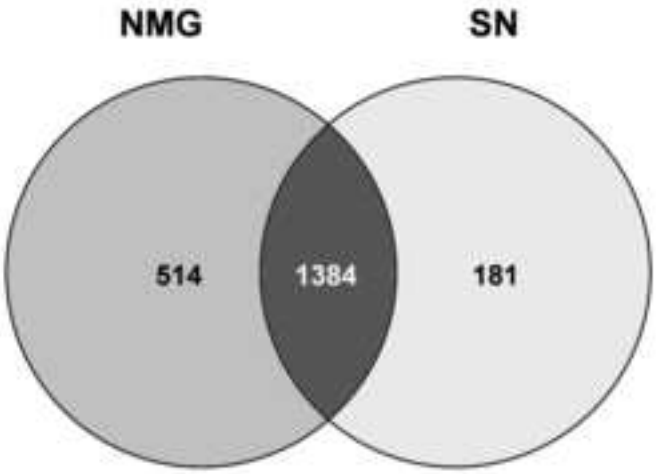
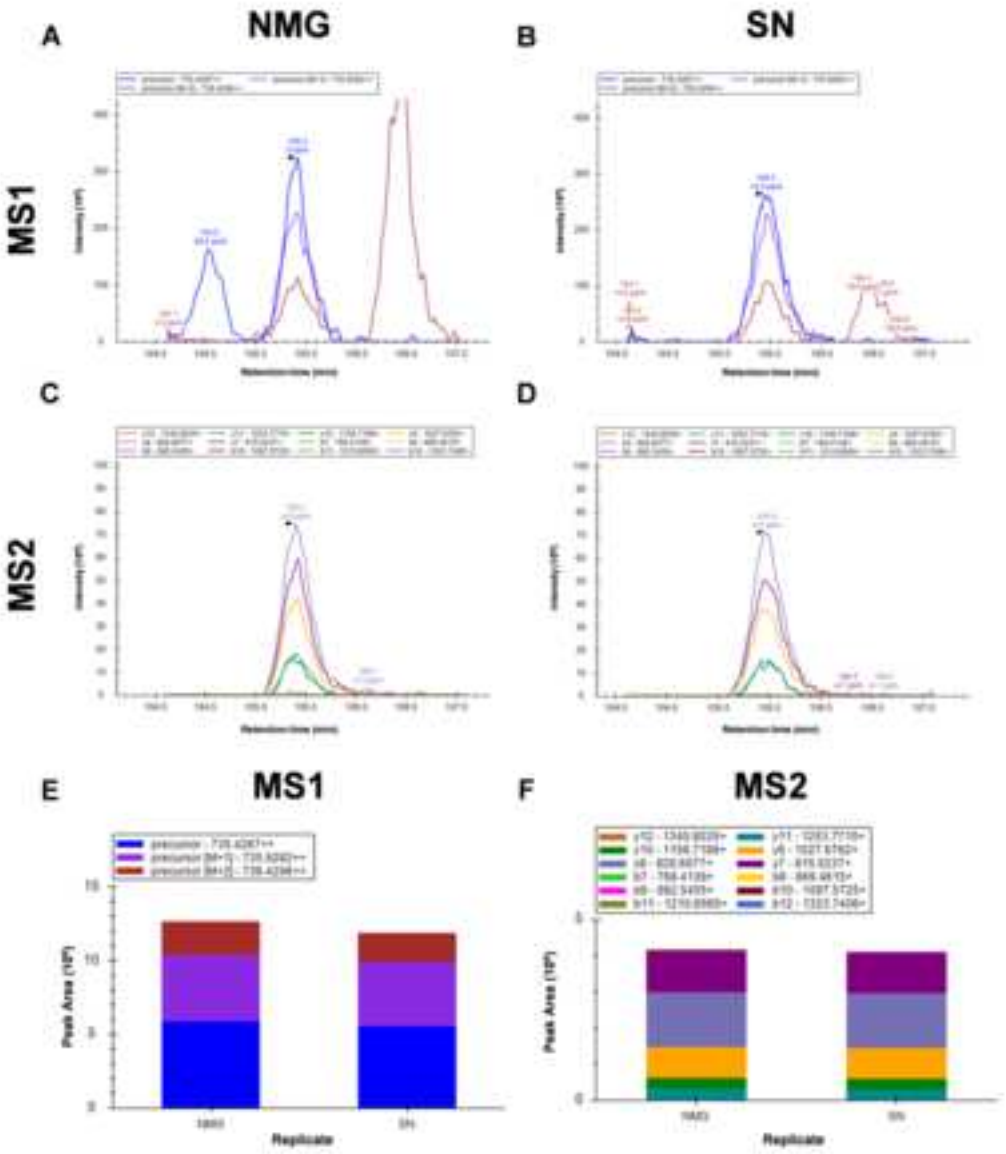


Figure 5

Click here to access/download;Figure;Figure5.tif



Rebuttal document

Laser microdissection-based protocol for the LC-MS/MS analysis of the proteomic profile of neuromelanin granules

Dear Editor,

We want to thank you and the reviewers for the comments and recommendations concerning our manuscript “Laser microdissection-based protocol for the LC-MS/MS analysis of the proteomic profile of neuromelanin granules”. You can find responses to each of your comments down below. With this, we hope that the resubmitted version is acceptable for publication now.

Kind regards,

Maximilian Wulf, on behalf of all Co-Authors

Editorial comments:

Editorial Changes

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The manuscript was proofread thoroughly for spelling and grammar issues.

2. Please revise the following lines to avoid previously published work: 108, 111-117, 123, 149, 202-206, 216, 226, 261.

All lines were revised accordingly.

3. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

All speeds were converted with one exception (p.7 line 208), as no transformation into g is possible for the used thermomixer.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example ThermoFisher Scientific, etc.

We removed commercial language in the context of software and instrumentation names.

5. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.
Step 1.2: Where was the stainless-steel knife installed?

For clarification, we revised the phrase “Clean the stainless steel knife with 70% ethanol and install it into the blade holder.” (line 109)

Step 1.5: What is section medium? Please define its composition.

As we used a commercial frozen section medium, we can unfortunately not define its composition.

Step 1.7: What is the optimum orientation for the tissue holder?

We included the following sentence in the manuscript “Optimal orientation depends on the orientation of your tissue.” (line 119)

Steps 1.8 and 1.10 are the same. Were cutting settings adjusted twice? If yes, what were the settings in step 1.8?

We removed step 1.8 from the protocol, as an adjustment of the cutting settings is necessary only before sectioning of the area of interest.

Step 1.14: The anti-roll plate contained the membrane slide? If yes, please mention this.

No, the anti-roll plate does not contain the membrane slides. For clarification, we added a more detailed description in lines 128 and 129 “Cut a section of the tissue, open the Anti-roll Plate carefully, take a membrane slide and prevent tissue folding while placing the tissue section on the membrane slide.”

Step 2.16: Please provide sonication settings used.

We added the used sonification settings (45kHz; line 198).

Step 3.7: How much trypsin was added?

We added 0.1 µg of trypsin per 1,000,000 µm² of tissue as stated in the NOTE belonging to former step 3.7 (now 3.6) “NOTE: For 1,000,000 µm², 0.1 µg of trypsin was found to be sufficient.”

Step 7.1: Please mention the proteins used in this study.

We addressed that comment by mentioning the protein cytoplasmic dynein 1 heavy chain 1, which was used in this study for PRM experiments “For our representative results, we selected cytoplasmic dynein 1 heavy chain 1, …” (line 308-309).

6. Please ensure that the table of materials contains all the reagents, equipment, glassware, etc. used in the study along with catalog number and company name.

From personal correspondence with one of the JoVE editors (Neethu Abraham), we got the information to only include those materials that were essential for the presented protocol. The included materials are essential, as used centrifuges, pipettes, mass spectrometer etc. could be exchanged.

7. Please include one line space between all step, substeps and notes in the protocol.

Done.

8. Please also include the following in the Discussion along with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique

We included all of these aspects in the last paragraph of the discussion (lines 434-455).

9. Figure 5: Please provide retention time unit.

The retention time unit is now provided in the labeling of the axis in figure 5.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

In this methodological protocol, Wulf et al. describe the complete workflow to characterise the molecular composition (proteins) of neuromelanin granules. The protocol is well-written covering from all experimental designs/settings to validation assays, including all parameters applied with all the softwares used. The detailed description of the manuscript makes it a robust protocol to be used not only by researchers with interest in neurological disorders but also by the scientific community with interest in the isolation and characterization of specific cells or aggregates in multiple biomedical contexts.

We thank Reviewer #1 for his comments to the presented protocol.

Reviewer #2:

Manuscript Summary:

very fine ms and protocol

We thank Reviewer #2 for his comments to the presented protocol.

Supplementary Table 1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);JoVe Supplementary Table 1.xlsx

MS 1 Scan properties

Detector type	Orbitrap
Orbitrap resolution	120000
Mass range	Normal
Use Quadrupole Isolation	yes
Scan range (m/z)	350-1400
RF Lens (%)	30
AGC target	Custom
Normalzed AGC target (%)	250
Maximum Injection Time Mode	Custom
Maximum Injection Time (ms)	80
Microscans	1
Data Type	Profile
Polarity	Positive
Source Fragmentation	no

MIPS	Peptide
Intensity	1.00E+04
Charge State	2-7
Include undetermined charge states	no

Dynamic exclusion

Exclude after n times	1
Exclusion duration (s)	30
Mass tolerance	ppm
Low	10
High	10
Exclude isotopes	yes

MS2 Properties

Isolation Mode	Quadrupole
Isolation Window (m/Z)	1.3
Isolation Offset	Off
Activation type	HCD
Collision Energy Mode	Fixed
HCD Collision Energy (%)	28
Detector Type	Orbitrap
Orbitrap Resolution	30000
Mass Range	Normal
Scan Range Mode	Auto
AGC target	Custom
Normalzed AGC target (%)	2000
Maximum Injection Time Mode	Custom
Maximum Injection Time (ms)	80
Microscans	1
Data Type	Profile

MS 1 Scan properties

Detector type	Orbitrap
Orbitrap resolution	60000
Mass range	Normal
Use Quadrupole Isolation	yes
Scan range (m/z)	350-1400
RF Lens (%)	30
AGC target	Custom
Normalzed AGC target (%)	250
Maximum Injection Time Mode	Custom
Maximum Injection Time (ms)	50
Microscans	1
Data Type	Profile
Polarity	Positive
Source Fragmentation	no

MS2 Properties

Isolation Mode	Quadrupole
Isolation Window (m/Z)	0.5
Activation type	HCD
Collision Energy Mode	Fixed
HCD Collision Energy (%)	28
Detector Type	Orbitrap
Orbitrap Resolution	60000
Mass Range	Normal
Scan Range Mode	Define m/z range
Scan Range (m/z)	100-2000
RF Lens (%)	30
AGC target	Custom
Normalzed AGC target (%)	2000
Maximum Injection Time Mode	Custom
Maximum Injection Time (ms)	200
Microscans	1
Data Type	Profile
Polarity	Positive
Source Fragmentation	No
Use EASY-IC	No
Loop Control	All
Dynamic Retention Time	Off
Time Mode	Start/End Time

MS 1 Scan properties

Detector type	Orbitrap
Orbitrap resolution	120000
Mass range	Normal
Use Quadrupole Isolation	yes
Scan range (m/z)	350-1400
RF Lens (%)	30
AGC target	Custom
Normalzed AGC target (%)	250
Maximum Injection Time Mod	Custom
Maximum Injection Time (ms)	50
Microscans	1
Data Type	Profile
Polarity	Positive
Source Fragmentation	no

MS2 Properties

Isolation Mode	Quadrupole
Isolation Window (m/Z)	0.5
Activation type	HCD
Collision Energy Mode	Fixed
HCD Collision Energy (%)	28
Detector Type	Orbitrap
Orbitrap Resolution	60000
Mass Range	Normal
Scan Range Mode	Define m/z range
Scan Range (m/z)	100-2000
RF Lens (%)	30
AGC target	Custom
Normalzed AGC target (%)	2000
Maximum Injection Time Mod	Custom
Maximum Injection Time (ms)	200
Microscans	1
Data Type	Profile
Polarity	Positive
Source Fragmentation	No
Use EASY-IC	No
Loop Control	All
Dynamic Retention Time	Off
Time Mode	Retention Time Window (3 min)

Parameter
Version
Date of writing
Include contaminants
PSM FDR
PSM FDR Crosslink
Protein FDR
Site FDR
Use Normalized Ratios For Occupancy
Min. peptide Length
Min. score for unmodified peptides
Min. score for modified peptides
Min. delta score for unmodified peptides
Min. delta score for modified peptides
Min. unique peptides
Min. razor peptides
Min. peptides
Use only unmodified peptides and
Modifications included in protein quantification
Peptides used for protein quantification
Discard unmodified counterpart peptides
Label min. ratio count
Use delta score
iBAQ
iBAQ log fit
Match between runs
Find dependent peptides
Fasta file
Decoy mode
Include contaminants
Advanced ratios
Fixed andromeda index folder
Combined folder location
Second peptides
Stabilize large LFQ ratios
Separate LFQ in parameter groups
Require MS/MS for LFQ comparisons
Calculate peak properties
Main search max. combinations
Advanced site intensities
Write msScans table
Write msmsScans table
Write ms3Scans table
Write allPeptides table
Write mzRange table
Write DIA fragments table

Write pasefMsmsScans table
Write accumulatedPasefMsmsScans table
Max. peptide mass [Da]
Min. peptide length for unspecific search
Max. peptide length for unspecific search
Razor protein FDR
Disable MD5
Max mods in site table
Match unidentified features
Epsilon score for mutations
Evaluate variant peptides separately
Variation mode
MS/MS tol. (FTMS)
Top MS/MS peaks per Da interval. (FTMS)
Da interval. (FTMS)
MS/MS deisotoping (FTMS)
MS/MS deisotoping tolerance (FTMS)
MS/MS deisotoping tolerance unit (FTMS)
MS/MS higher charges (FTMS)
MS/MS water loss (FTMS)
MS/MS ammonia loss (FTMS)
MS/MS dependent losses (FTMS)
MS/MS recalibration (FTMS)
MS/MS tol. (ITMS)
Top MS/MS peaks per Da interval. (ITMS)
Da interval. (ITMS)
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MS/MS deisotoping tolerance (ITMS)
MS/MS deisotoping tolerance unit (ITMS)
MS/MS higher charges (ITMS)
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MS/MS deisotoping tolerance (TOF)
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MS/MS tol. (Unknown)

Top MS/MS peaks per Da interval. (Unknown)
Da interval. (Unknown)
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MS/MS deisotoping tolerance (Unknown)
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Filming Location(s): Medizinisches Proteom-Center, Bochum
Producer: JoVE

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Author(s): M. Wulf, K. Burkovits-Boeddinghaus, P. Sommer, K. Schork, M. Eisenacher, P. Riederer, M. Gerlach, S. Kösters, B. Eggers & K. Marcus

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