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Bulk Proteomics (DIA-MS) of Human Dorsal Root Ganglion

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Allison Barry[1,2], Theodore Price[1], Manuela Schmidt[2]

[1]University of Texas Dallas; [2]University of Vienna

PRECISION Human Pain ...

Allison Barry
University of Texas Dallas

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We use this protocol and it's working

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Abstract

In this protocol, we describe how to perform bulk proteomics on frozen human dorsal root ganglia (hDRG) tissue from donors from tissue preparation to data-independent acquisition mass spectrometry (DIA-MS).

Materials

	Equipment	Company	Location
	LoBind Protein Eppendorf tube	Eppendorf	Hamburg, DE
	Bioruptor Pico	Diagenode Seraing	Belgium
	ThermoMixer C	Eppendorf	Hamburg, DE
	NanoPhotometer N60	Implen	Munich, DE
	DynaMag™-2 Magnet	ThermoFisher	
	timsTOF HT	Bruker	Bremen, DE
	C18 trap column (1 mm x 5 mm)	ThermoFisher	
	AuroraTM ULTIMATE column (25 cm × 75 µm)	IonOpticks	Australia

Table: Required equipment.

	Component	Provider	Identifier	Company RRID
	Tris 1 M	Accugene/Avantor	CAT# 733-1653	RRID:SCR_000377
	Sera-Mag SpeedBead beads	Cytiva	CAT# 65152105050250, CAT# 45152105050250 at a 1:1 mix	RRID:SCR_023581
	10× PBS	Fisher Scientific	CAT# 11594516	RRID:SCR_008452
	Acetonitrile	Fisher Scientific	CAT# 10001334	RRID:SCR_008452
	Formic acid	Fisher Scientific	CAT# 15658430	RRID:SCR_008452
	Glycerol	Fisher Scientific	CAT# 10021083	RRID:SCR_008452
	Trypsin/Lys-C	Promega	CAT# V5073	RRID:SCR_006724
	Acetone	Sigma-Aldrich	CAT# 1000201000	RRID:SCR_008988
	Ammonium bicarbonate	Sigma-Aldrich	CAT# 09830-500G	RRID:SCR_008988
	Dithiothreitol (DTT) 1 M	Sigma-Aldrich	CAT# 43816	RRID:SCR_008988

	Component	Provider	Identifier	Company RRID
	Ethanol	Sigma-Aldrich	CAT# 1117272500	RRID:SCR_008988
	Iodoacetamide	Sigma-Aldrich	CAT# I1149	RRID:SCR_008988
	Water MS grade	Sigma-Aldrich	CAT# 1.15333.1000	RRID:SCR_008988

Table: Required Chemicals.

	Software	Version	Citation	RRID
	R	>4.2	https://www.r-project.org/	RRID:SCR_001905
	Bioconductor		http://www.bioconductor.org/	RRID:SCR_006442
	limma		Ritchie et al., 2015	RRID:SCR_010943
	clusterProfiler		Wu et al., 2021	RRID:SCR_016884
	DIA-NN	1.8.1	Demichev et al., 2020	RRID:SCR_022865

Table: General software for processing

Before start

Appropriate PPE includes lab coat and gloves. Proper safety precautions for work with and disposing off human tissue must be followed. Dissection tools should be cleaned and sterilized prior to starting.

Ensure you have all materials listed in the "Materials" section.

Tissue Harvesting

1 Extended tissue procurement methods are described in dx.doi.org/10.17504/protocols.io.kqdg32qr1v25/v1, see notes on fresh-frozen samples from steps 1-35

1.1 Surgically excised lumbar (L1-L5) human dorsal root ganglia (hDRGs) are obtained from organ donors at Southwest Transplant Alliance (STA) within 4h post cross-clamp

1.2 Each hDRG is placed in an epitube, labelled, and buried directly in powdered dry ice prior to transport (on dry ice) to a -80°C for storage

1.3 Here, tissue was then shipped on dry ice to another facility for proteome-specific processing, and again stored at -80°C

Tissue cleaning and lysis

15h 20m

2 12-24 hours prior to dissection, tissue is transferred to -20°C

12h

3 *Chill chemicals:* chill 100% acetone at -20°C and 80% EtOH at 4°C for later use

4 *Prepare lysis buffer*: 2% SDS, 100mM Tris, 5% glycerol, 10mM DTT and 1x protease inhibitor cocktail

4.1 Add 500 µl lysis buffer to LoBind Protein Eppendorf tubes, with 1-4 tubes per sample depending on tissue amount

5 For each DRG, tissue is thawn on ice for ~15 min prior to dissection (also on ice).

15m

Rinse 1x with cold PBS and dissected to remove the surrounding connective tissue and fat. In each ganglia, a nerve root extending from the core ganglia was identified. This is presumed to contain fewer/no neuronal somata and is transected crudely with a scalpel for separate processing

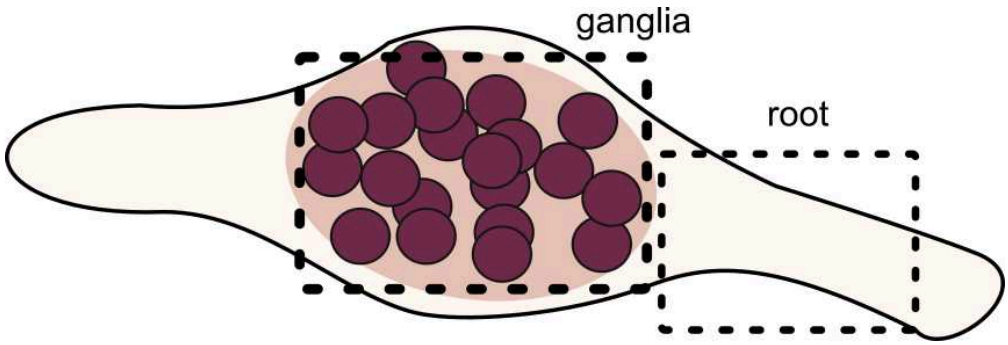

Fig 1. Dissection schematic

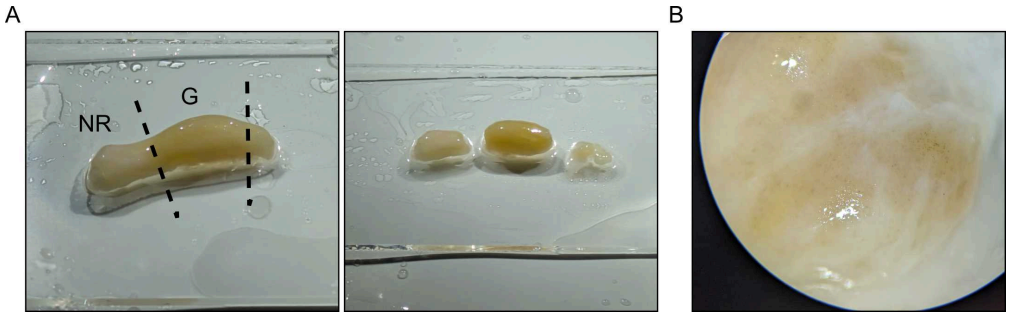

Fig 2. Photographs of clean hDRG. A. DRG before (left) and after (right) transecting the nerve root (NR) from the ganglia (G). B. Low magnification photo of the pigmentation aggregates visible within the ganglia (view down dissecting microscope).

6 Due to the size of the tissue each hDRG can be cut into up multiple pieces (typically 1-4) and subsequently, each piece is cut in smaller pieces to increase the surface area for lysis. All small pieces of one big cut can then be transferred to a pre-filled eppendorf tube (step 5)

7 Sonicate tissue for 15 min (cycles of 30 sec ON and 30 sec OFF, 4°C, low frequency) in a Bioruptor Pico 15m

8 Incubate for 15 min at 70°C, 1500 rpm in a ThermoMixer C 15m

9 Centrifuge for 5 min with 10000 × g at room temperature (RT) to pellet cell debris 5m

10 Combine supernatant (~480 µl) with 5x sample volume 100% acetone (pre-chilled) and incubate at -20°C for 2.15-2.45 h to precipitate the proteins. 2h 30m

NOTE: Supernatant can be split into 2 eppendorfs per sample if there are volume constraints

11 Centrifuge for 30 min at 14000 × g, RT 30m

12 Remove supernatant and wash pellet with ice-cold 80% EtOH

13 Centrifuge for 30 min at 14000 × g, RT 30m

14 Remove EtOH and air dry pellets for 20 min 20m

15 Resolubilise pellet in lysis buffer by incubation for 10 min at 70°C, 1500 rpm (ThermoMixer C) 10m

16 Measure total protein concentration per eppendorf at 280 nm with a NanoPhotometer N60

17 If an hDRG was processed in multiple tubes due to the size, these can be merged by equal volume into a single sample. If combined, measure final concentration again.

18 Samples can now be flash-frozen and stored at -20°C

Protein clean-up and digestion

19 Perform as described in Xian et al., 2022, protein clean-up and digestion is based on the single-pot, solid-phase-enhanced sample preparation (SP3) method from Hughes et al. (Hughes et al., 2019). NOTE: Hughes et al., also contains a supplemental video outlining each SP3 step.

20 *Reduction:* Combine 50 µg total protein of each sample with 5 mM final concentration dithiothreitol (DTT) from a 1M stock for 30 min at 60°C 30m

21 *Alkylation:* mix with 20 mM iodoacetamide (IAA) for 30 min in the dark at RT 30m

22 Dilute 1M DTT stock to a final concentration of 5 mM to quench any remaining DTT for 15 min at RT — 15m

23 Add 10 µL of pre-mixed Sera-Mag SpeedBead beads (1:1) to each sample, as well as one sample volume of absolute EtOH to initiate protein binding to the beads

24 Incubate in a ThermoMixer C for 5 min with 1000 rpm agitation at 24°C — 5m

25 Collect beads on a magnetic rack (2 min) and discard the supernatant — 2m

26 Rinse beads 3x with 500 µL of 80% EtOH

27 Reconstitute beads in 50 µL ammonium bicarbonate (50 mM, pH 8) containing 2 µg Trypsin/Lys-C. Incubate for 18 h at 37°C with 950 rpm agitation to digest proteins — 18h

28 *Peptide clean-up:* acetonitrile (ACN) was added to each sample to a final concentration of 95%. Samples were incubated for 8 min at RT and then the beads were collected on a magnetic rack (2 min) — 10m

29 *Peptide clean-up:* Discard the supernatant and wash beads with 900 µL of 100% ACN

30 *Peptide elution:* add 40 µL LC-MS grade H_2O

31 Measure peptide concentration at 205 nm with a NanoPhotometer N60 (Implen)

32 Add formic acid (FA) to a final concentration of 0.1% and store at -20°C until further use

LC-MS/MS

33 Perform peptide analysis via NanoElute LC (Nanoflow reversed-phase liquid chromatography (Nano-RPLC) performed on a NanoElute2 system) coupled with a hybrid TIMS quadrupole TOF mass spectrometer (timsTOF HT, Bruker Daltonik) via a CaptiveSpray ion source. *The following parameters are used:*

33.1 Mobile phase A: 100% water, 0.1% FA.
Mobile phase B: 100% acetonitrile (ACN), 0.1% FA

500 ng of peptides

Load peptides onto C18 trap column (1 mm x 5 mm, ThermoFisher), further separating over a 90 min gradient on an AuroraTM ULTIMATE column (25 cm × 75 µm) packed with 1.6 µm C18 particles (IonOpticks, Fitzroy, Australia).

Flow rate = 250 nL/min, except for the last 7 minutes, where the flow rate accelerates to 400 nL/min

The mobile phase B increases linearly from 2 to 20% in the first 60 minutes, followed by another linear increase to 35% within 22 minutes and a steep increase to 85% in 0.5 min. Then, a flow rate switches to 400 nL/min in 0.5 min and is maintained for 7 minutes to the end of the gradient to elute all hydrophobic peptides.

33.2 Analyze samples in data-independent acquisition (DIA) mode coupled with parallel accumulation serial fragmentation (PASEF). Precursors with m/z between 350 and 1200 are defined in 13 cycles (either 2 or 3 quadrupole switches per cycle) containing 34 ion mobility steps within the ion mobility range of 0.65 – 1.35 (1/k0) with fixed isolation window of 25 Th in each step.

The acquisition time of each DIA-PASEF scan is set to 100 ms, resulting in a total cycle time of around 1.48 sec.

The collision energy is ramped linearly from 65 eV at 1/k0 = 1.6 to 20 eV at 1/k0 = 0.6.

DIA-PASEF data analysis

34 DIA-NN v1.8.1 (Demichev et a., 2020) is run with a predicted library search to process raw output files (`.d`) against the human proteome (UP000005640) with match-between-runs (MBR) enabled

34.1
```
#!/bin/bash
#SBATCH -J diann_hdrg
#SBATCH -N 1
#SBATCH --cpus-per-task=128

module purge

DATA=`\path\to\data\directory`
OUT=`\path\to\output\directory`
```

```
./bin/diann-1.8.1 --dir $DATA \
      --gen-spec-lib --fasta-search --use-quant \
      --predictor \
      --fasta hdrg/UP000005640_validated.fasta \
      --threads 128 --verbose 1 --qvalue 0.01 --matrices \
      --min-corr 2.0 --corr-diff 1.0 --time-corr-only \
      --min-fr-mz 100 --max-fr-mz 1700 \
      --out $OUT/report.tsv \
      --out-lib $OUT/report-lib.tsv \
      --cut K*,R* --missed-cleavages 2 --min-pep-len 5 --max-pep-len 52 \
      --min-pr-mz 350 --max-pr-mz 1200 --min-pr-charge 2 --max-pr-charge 4 \
      --var-mods 3 --var-mod UniMod:35,15.994915,M --var-mod UniMod:1,42.010565,*n
\
      --monitor-mod UniMod:1 --rt-profiling --reanalyse --met-excision \
      --pg-level 1 --no-ifs-removal
```

35 MaxLFQ per peptide and gene group is calculated in R using the diann package:

35.1
```
library(diann)
library(dplyr)

df ← diann_load("./report.tsv")

precursors ← diann_matrix(df, q = 0.01)

peptides.maxlfq ← diann_maxlfq(df[df$Q.Value <= 0.01 & df$PG.Q.Value <= 0.01,],
                        sample.header = "Run",
                        group.header="Stripped.Sequence",
                        id.header = "Precursor.Id",
                        quantity.header = "Precursor.Normalised")

gene.groups ← diann_maxlfq(df[df$Q.Value <= 0.01 & df$GG.Q.Value <= 0.01,],
                       sample.header = "Run",
                       group.header="Genes",
                       id.header = "Precursor.Id",
                       quantity.header = "Precursor.Normalised")
```

36 Downstream processing can then be performed on a matrix of gene group expression (eg. GSEA via clusterProfiler, differential expression testing with limma)

Protocol references

Demichev, V., Messner, C.B., Vernardis, S.I. *et al.* DIA-NN: neural networks and interference correction enable deep proteome coverage in high throughput. *Nat Methods* **17**, 41–44 (2020). https://doi.org/10.1038/s41592-019-0638-x

Hughes CS, Moggridge S, Müller T, Sorensen PH, Morin GB, Krijgsveld J. Single-pot, solid-phase-enhanced sample preparation for proteomics experiments. Nat Protoc. 2019 Jan;14(1):68-85. doi: 10.1038/s41596-018-0082-x. PMID: 30464214.

Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK. limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res. 2015 Apr 20;43(7):e47. doi: 10.1093/nar/gkv007. Epub 2015 Jan 20. PMID: 25605792; PMCID: PMC4402510.

Xian, F., Sondermann, J.R., Gomez Varela, D., Schmidt, M. Deep proteome profiling reveals signatures of age and sex differences in paw skin and sciatic nerve of naïve mice. *eLife* 11:e81431 (2022) https://doi.org/10.7554/eLife.81431

Wu, Tianzhi, et al. "clusterProfiler 4.0: A universal enrichment tool for interpreting omics data." *The innovation* 2.3 (2021).