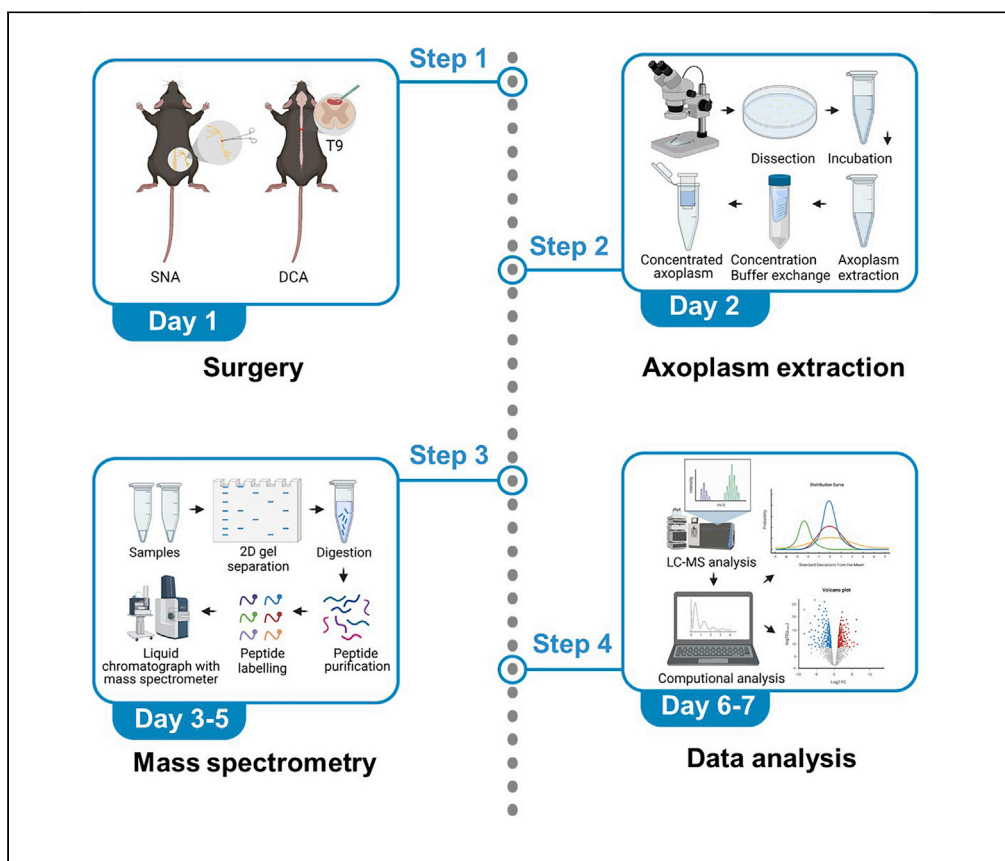# STAR Protocols

Protocol

Purification of mouse axoplasmic proteins from dorsal root ganglia nerves for proteomics analysis

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Highlights

Detection of
axoplasmic protein
expression in the
mouse after nerve
injury

Axoplasm protein
extraction and
concentration from
the sciatic nerve and
dorsal roots

Mass spectrometry
analysis of
axoplasmic protein
expression

The study of neuronal signaling *ex vivo* requires the identification of the proteins that are represented within the neuronal axoplasm. Here, we describe a detailed protocol to isolate the axoplasm of peripheral and central axonal branches of sciatic dorsal root ganglia neurons in mice. The axoplasm is separated by 2D gel and digestion followed by proteomics analysis with MS/MS-LC. This protocol can be applied to dissect the axoplasmic protein expression signatures before and after a sciatic nerve or a spinal cord injury.

STAR Protocols

Protocol

Purification of mouse axoplasmic proteins from dorsal root ganglia nerves for proteomics analysis

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SUMMARY

The study of neuronal signaling *ex vivo* requires the identification of the proteins that are represented within the neuronal axoplasm. Here, we describe a detailed protocol to isolate the axoplasm of peripheral and central axonal branches of sciatic dorsal root ganglia neurons in mice. The axoplasm is separated by 2D gel and digestion followed by proteomics analysis with MS/MS-LC. This protocol can be applied to dissect the axoplasmic protein expression signatures before and after a sciatic nerve or a spinal cord injury.

For complete details on the use and execution of this protocol, please refer to Kong et al. (2020).

BEFORE YOU BEGIN

Unlike other cells, neurons are characterized by a long cytoplasmic process called axoplasm, where information is transported anterogradely and retrogradely in the form of protein trafficking along the axons between the cell body and the synapse, including across considerable distances. Therefore, the axonal cytoplasm contains a wealth of unique biological information on the state of neuronal activity and function that underpins neuronal communication in both physiological and pathological conditions. Given that axons are tightly packed within the connective tissue and are surrounded by glial cells, achieving proper enrichment and purity of axoplasmic proteins ex vivo is not trivial. Investigating protein changes following axoplasmic protein extraction is critical for studying neuronal specific cell biology and to distinguish between neuronal intrinsic and extrinsic signaling mechanisms that underpin repair and regeneration as following an axonal injury.

While axonal regeneration and partial functional recovery occur when an injury lies in the peripheral nervous system (PNS), these fail when axons are injured within the central nervous system (CNS) such as in the spinal cord. Dorsal root ganglia (DRG) contain pseudo-unipolar neurons that extend one branch into the peripheral nervous system such as the sciatic nerve for example and one branch into the dorsal column of the spinal cord, allowing researchers to compare the molecular mechanisms associated with the differential regenerative ability of the PNS versus CNS axons that share the same cell body. Several molecular mechanisms supporting this differential regenerative ability have been reported, including epigenetic regulators (Cho et al., 2013; Ziv and Spira, 1995), calcium signaling leading to the phosphorylation of STAT3 (Ben-Yaakov et al., 2012), and ERK (Perlson et al., 2005), and the dephosphorylation of HDAC3 (Hervera et al., 2019), which in turn initiate the regeneration program of DRG neurons by activating transcription factors such as ATF3, CREB, and c-JUN among others (Herdegen et al., 1992; Hervera et al., 2019; Lindwall and Kanje, 2005). However, the molecular and signaling mechanisms reflected by the protein content in the axoplasm remain largely
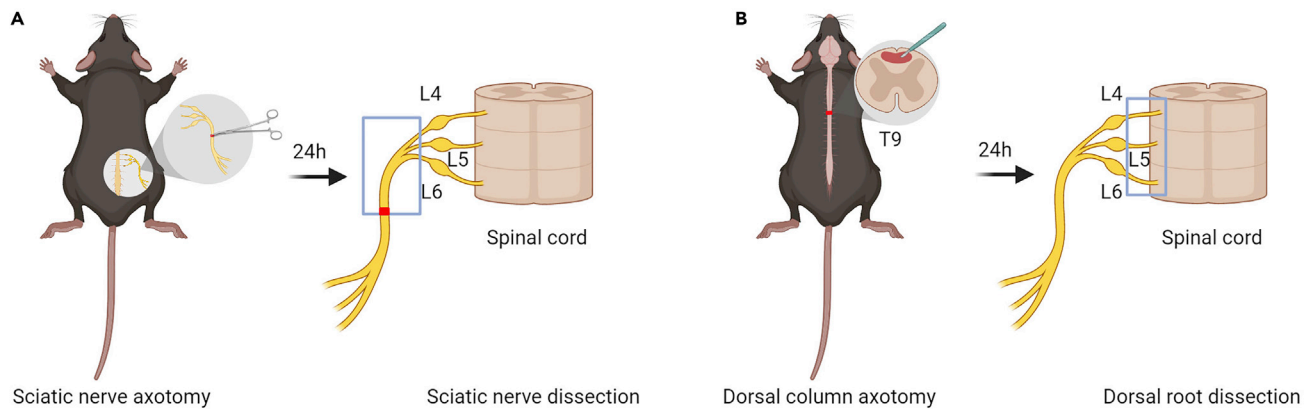
Figure 1. Schematic of experimental design
(A) Diagrams of sciatic nerve injury and dissection. The blue box shows the sciatic nerve segment dissected for axoplasm extraction.
(B) Diagrams of T9 dorsal column injury and dorsal root dissection. The blue box shows the L4-L6 DRG dorsal roots dissected for axoplasm extraction.

elusive, with the notable exception of selected studies in the rat (Michaelevski et al., 2010a, 2010b). Studies documenting protein axoplasmic analysis in the mouse from central projecting sciatic DRG roots including after a spinal cord injury as well as the direct comparison with the peripheral axons after sciatic injury have remained elusive until our recent work (Kong et al., 2020).

Here we describe a protocol that portrays the isolation, purification, and enrichment of axoplasmic proteins extracted from both sciatic nerve and sciatic dorsal roots including following sciatic nerve or spinal cord injury in mice coupled with protein expression profiling by performing mass spectrometry. Feedback from Dr. Fanizilber's laboratory was instrumental to establish this protocol. The study design includes four experimental groups and conditions as follows: Sham, sciatic nerve axotomy (SNA), laminectomy (Lam), and spinal dorsal column axotomy (DCA). For each condition, three biological replicates are prepared, requiring pooling axoplasmic extracts from ten or fifteen mice per replicate from the sciatic nerve or dorsal roots respectively. Sciatic nerve injury is performed on the mid-thigh level and spinal cord injury is performed at the ninth thoracic level (Figure 1). Mice are sacrificed 24h after surgery for the axoplasm purification, enrichment, and mass spectrometry. Since the tissue dissection of one biological replicate takes several hours, we recommend completing the surgery for each biological replicate one day before the axoplasm extraction (10 mice of the sciatic nerve, or 15 mice of the dorsal roots). As a result, the collection of axoplasm for the above experimental design will be completed in 12 days. All animal surgical and experimental procedures are performed in accordance with the UK Animal Scientific Procedures Act (1986) and approved by the ethical committee of Imperial College London. Given the standard nature of the protocols, we do not provide the experimental details for sciatic nerve and spinal cord injuries. For more information on the surgery protocols, please refer to our recent publication (Kong et al., 2020).

Preparation of tools and microscope for the dissection

⏱ Timing: ∼ 10 min

1. Prepare a dissection microscope for the dissection as illustrated in Figure 2A. Clean the base surface of the microscope with 70% ethanol.
2. Prepare dissection tools as shown in Figure 2B for the dissection.

Preparation of solutions for axoplasm purification and concentration

⏱ Timing: ∼30 min
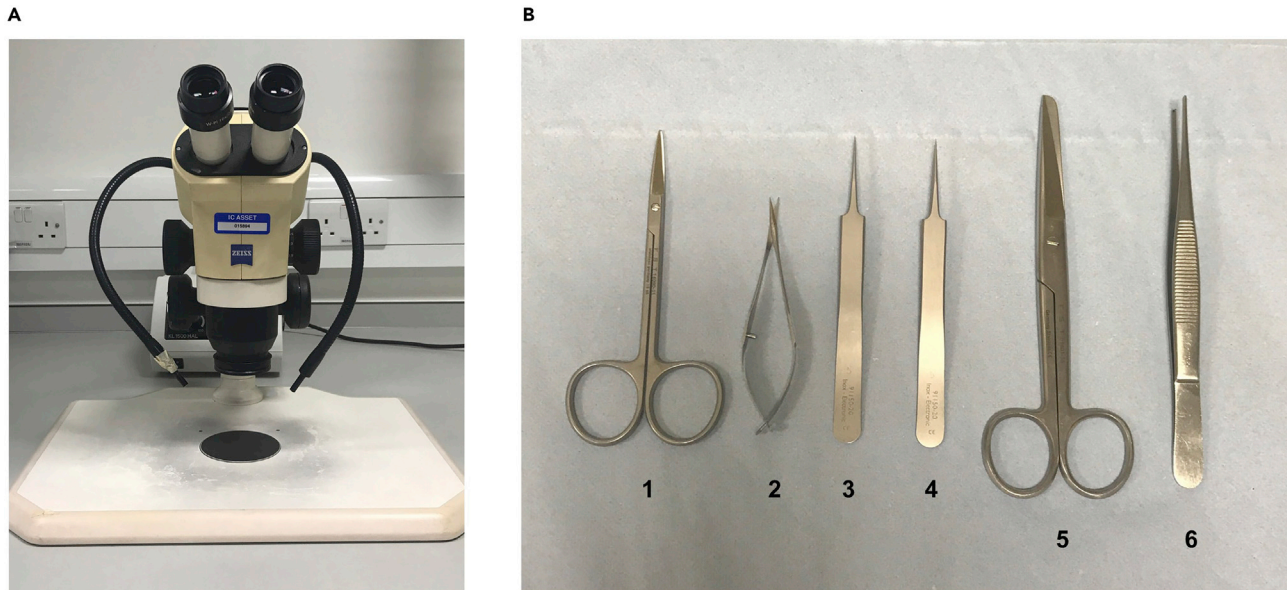
Figure 2. Preparation of dissection experiment.

(A) Zeiss microscope with a light source for dissection

(B) Surgical tools used for sacrificing animals and dissection. Tools from the left to the right: 1, Hardern Fine Scissors, used for cutting muscles to expose sciatic nerve; 2, Student Vannas Spring Scissors, used to cut the ventral vertebrae and the tissues attached to the nerves, as well as to dissect sciatic nerve and dorsal roots; 3 and 4, Student Dumont #5 Forceps, used for sciatic nerve, dorsal root, and DRG dissection; 5 (Student Surgical Scissors) and 6 (Tissue Forceps with teeth): used for sacrificing mice.

3. Prepare protease inhibitor and phosphatase inhibitor solutions
   a. 50× protease inhibitor (prepare fresh): dissolve one protease inhibitor tablet in 1 mL ddH$_2$O and keep it on ice.
   b. 10× phosphatase inhibitor (prepare fresh): dissolve three phosphatase inhibitor tablets in 3 mL ddH$_2$O and keep it on ice.
4. Prepare all the other solutions used for axoplasm purification and concentration following the instructions in the recipe tables (Materials and equipment).

Preparation of solutions for sample preparation, peptide extraction, and purification

⏱ Timing: ~1 h

5. Prepare solutions for sample preparation, peptide extraction, and purification
   a. 25 mM NH$_4$HCO$_3$ (500 mL): dissolve 0.99g NH$_4$HCO$_3$ into 500 mL ddH$_2$O. Keep at 20°C–22°C for long-term storage.
   b. 50 mM NH$_4$HCO$_3$, pH 8.0 (500 mL): dissolve 1.98g NH$_4$HCO$_3$ into 400 mL ddH$_2$O, adjust PH to 8.0, add ddH$_2$O to 500 mL. Keep at 20°C–22°C for long-term storage.
   c. Destain buffer (10 mL, prepare fresh): add 5 mL 100% ethanol into 5 mL 25 mM NH$_4$HCO$_3$ to make 50% ethanol concentration in the solution. Keep at 20°C–22°C.
   d. Reduction buffer (10 mL, prepare fresh): 0.2 mL 500 mM DTT in 9.8 mL 50 mM NH$_4$HCO$_3$, pH 8.0. Keep at 20°C–22°C.
   e. Alkylation buffer (10 mL, prepare fresh and use immediately, protect from light): dissolve 0.09 g Iodoacetamide in 10 mL 50 mM NH$_4$HCO$_3$, pH 8.0. Keep at 20°C–22°C.
   f. 50mM Triethylammonium bicarbonate buffer, pH 8.0 (100 mL, prepare fresh): 5 mL 1M triethylammonium bicarbonate buffer, pH 8.0 in 95 mL ddH$_2$O. Keep at 4°C prior to use.
   g. Trypsin digestion solution (160 µL for one sample, prepare fresh): dissolve 1 µg trypsin in 160 µL 50mM triethylammonium bicarbonate buffer, pH 8.0. Keep it on ice prior to use.

h. 30% Acetonitrile (100 mL): add 30 mL acetonitrile in 70 mL ddH$_2$O. Keep at 20°C–22°C for long-term storage.

6. Prepare all the other solutions for sample preparation, peptide extraction, and purification following the instructions in the recipe tables (Materials and equipment).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
1X PBS	Thermo Fisher Scientific	Cat#14190144
10X PBS	Thermo Fisher Scientific	Cat#14200075
Protease inhibitor	MERCK	Cat#04693116001
Phosphatase inhibitor	MERCK	Cat#04906837001
ddH$_2$O	MERCK	Cat#W3500
Tween20	MERCK	Cat#P2287
Urea	MERCK	Cat#U6504
Thiourea	MERCK	Cat#T7875
Tris base	MERCK	Cat#T1503
Pierce™ 20X TBS Buffer	Thermo Fisher Scientific	Cat#28358
NuPAGE™ LDS Sample Buffer	Thermo Fisher Scientific	Cat#NP0007
NuPAGE™ Sample Reducing Agent (10X)	Thermo Fisher Scientific	Cat#NP0004
10% NuPAGE Bis-Tris gel	Thermo Fisher Scientific	Cat#NP0301BOX
NuPAGE™ MES SDS Running Buffer (20X)	Thermo Fisher Scientific	Cat#NP000202
Acetic acid	MERCK	Cat#A6283
Methanol	MERCK	Cat#646377
Colloidal Blue Staining Kit	Thermo Fisher Scientific	Cat#LC6025
NH$_4$HCO$_3$	MERCK	Cat#A6141
Ethanol	MERCK	Cat#51976
Acetonitrile	MERCK	Cat#34998
C18 StageTips	Thermo Fisher Scientific	Cat#87784
Pierce™ Trypsin Protease, MS Grade	Thermo Fisher Scientific	Cat#90057
Triethylammonium bicarbonate buffer	MERCK	Cat#T7408
Iodoacetamide	MERCK	Cat#I1149
Pierce™ Formic Acid, LC-MS Grade	Thermo Fisher Scientific	Cat#28905
Pierce™ 0.1% Formic Acid (v/v) in Water, LC-MS Grade	Thermo Fisher Scientific	Cat#85170
Experimental models: Organisms/strains		
C57BL/6J mouse (male, 8-10 weeks old)	Charles River	Strain code: 632
Software and algorithms		
R	https://www.r-project.org/	N/A
Deposited data		
Raw axoplasm proteomics data	Kong et al., 2020	ProteomeXchange Consortium; accession code: PXD013297
Codes used to create histograms	Kong et al., 2020	https://github.com/intgenomics/191015.proteomics_analysis
Codes used to create volcano plots	This paper	https://github.com/gitgpk/Star-Protocols
Other		
Amicon® Pro Purification System with Ultra-0.5 Device	MERCK	Cat#ACS500312
Falcon 50mL Conical Centrifuge Tubes	Fisher Scientific	Cat#10788561
Falcon 15mL Conical Centrifuge Tubes	Fisher Scientific	Cat#11507411
Eppendorf® Protein LoBind tubes, 1.5mL	MERCK	Cat#EP0030108116
Eppendorf® Protein LoBind tubes, 2.0mL	MERCK	Cat#EP0030108132
Corning® non-treated 3.5 cm culture dishes	MERCK	Cat#CLS430588
Thermo Scientific™ Multifuge X3R	Fisher Scientific	Cat#15233457
Vacufuge plus - Centrifuge Concentrator	Eppendorf	Cat#022820001
Eppendorf™ 5427R Centrifuge	Fisher Scientific	Cat#15234526

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Zeiss Stemi 2000C microscope	Fisher Scientific	Cat#11895143
Stuart SRT6 Roller mixer	MERCK	Cat#Z671711
Q Exactive Plus mass spectrometer	Thermo Fisher Scientific	Cat#IQLAAEGAAPFALGMBDK
EASY-nLC 1000 Liquid Chromatograph	Thermo Fisher Scientific	Cat#LC120
Hardened Fine Scissors	Fine Science Tools	Cat#14090-11
Student Vannas Spring Scissors	Fine Science Tools	Cat#91500-09
Student Dumont #5 Forceps	Fine Science Tools	Cat#91150-20
Student Surgical Scissors	Fine Science Tools	Cat#91401-14
Tissue Forceps with teeth	Fine Science Tools	Cat#11023-14

MATERIALS AND EQUIPMENT

0.2× PBS solution (prepare fresh)

Reagent	Final concentration	Amount
1×PBS	0.2×	4 mL
Protease inhibitor (50×)	1×	0.4 mL
Phosphatase inhibitor(10×)	1×	2 mL
ddH$_2$O	n/a	13.6 mL
Total	n/a	20 mL

Note: Keep it on ice.

1× PBS solution (prepare fresh)

Reagent	Final concentration	Amount
10×PBS	1×	0.5 mL
Protease inhibitor (50×)	1×	0.1 mL
Phosphatase inhibitor(10×)	1×	0.5 mL
ddH$_2$O	n/a	3.9 mL
Total	n/a	5 mL

Note: Keep it on ice.

Denaturation buffer

Reagent	Final concentration	Amount
Urea	6 M	3.6 g
Thiourea	2 M	1.52 g
10mM Tris-Hcl pH 8.0	10 mM	10 mL
Total	n/a	10 mL

Note: After preparation, aliquot 1 mL into each tube, and store at −20°C for long-term storage.

TBST solution

Reagent	Final concentration	Amount
20× TBS	1×	0.5 mL
Tween 20	1%	0.1 mL
ddH$_2$O	n/a	9.4 mL
Total	n/a	20 mL

Note: Keep at 20°C–22°C for long-term storage. Pre-cool it on ice prior to use.

Gel fixation solution		
Reagent	Final concentration	Amount
Acetic acid	7%	7 mL
Methanol	40%	40 mL
ddH$_2$O	n/a	53 mL
Total	n/a	100 mL

Note: Store at 20°C–22°C for long-term storage.

Peptide elution buffer		
Reagent	Final concentration	Amount
Acetonitrile	80%	0.8 mL
Formic Acid	0.1%	1 μL
ddH$_2$O	n/a	199 μL
Total	n/a	1 mL

*Note:* Store at 2°C–8°C for long-term storage.

STEP-BY-STEP METHOD DETAILS
Sciatic nerve dissection

⏱ Timing: ~ 2 h

The steps of the sciatic nerve dissection 24 h after sciatic nerve injury are described in this section.

1. Sacrifice mouse by cervical dislocation 24 h after sciatic nerve injury.
2. Spray the entire body with 70% ethanol and remove skin and internal organs to make dissection easier.
3. Transfer the clean mouse body to a foam board and fix the fore and hind limbs on the board with four bent 20g needles keeping the back of the mouse onto the board.
4. Dissect the tissues under the dissection microscope.
   a. Using the student vannas spring scissors, carefully cut the ventral vertebrae and expose the spinal cord using forceps.
   b. L4-L6 sciatic DRG are located in the intervertebral foramen, carefully dissect these DRG by cutting the branches that are connected to them. Next, collect them in 1 mL 1× PBS with protease and phosphatase inhibitors in a 1.5 mL Eppendorf tube on ice for other experiments.
   c. Transpose the mouse body with its back upwards and fix it on the board again.
   d. Open the sciatic nerve wound with the hardened fine scissors and expose the injured sciatic nerve carefully with forceps.
   e. Remove the attached tissues as well as a very thin nerve near the main branch.
   f. Carefully grab the injured end of the proximal segment of the sciatic nerve with forceps and pull it along the spine.
   g. Once the nerve is pulled out as shown in Figure 3A, immediately place it in a 3.5 cm cell culture dish with 2 mL 0.2× PBS with protease and phosphatase inhibitors on ice.
   h. Collect 10 sciatic nerves from 5 mice in one 3.5 cell culture dish, therefore preparing 2 dishes for 10 mice.
5. Collect sciatic nerves from animals that have received the sham injury.
   a. Cut the nerves with the microdissection scissor in the wound site.
   b. Pull it out carefully with forceps after tissue clearance.
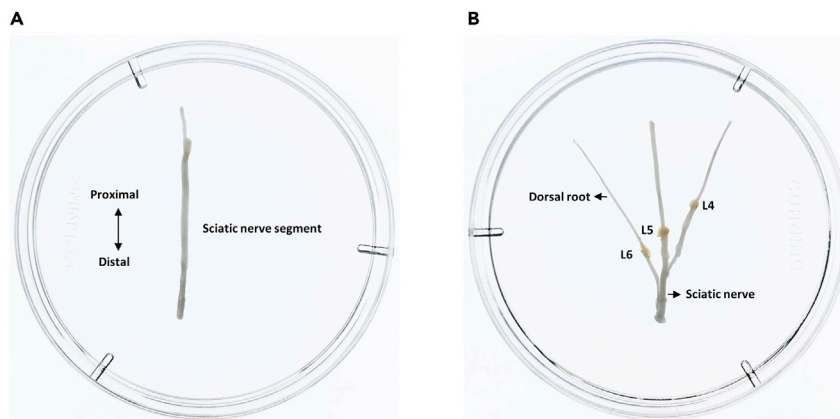
Figure 3. Sciatic nerve and sciatic dorsal roots dissection
(A and B) Dissected sciatic nerve segment (A) and sciatic dorsal roots attached with L4-L6 DRG (B)

   c. The length of the nerve should be the same as that collected from the mice who had SNA injuries. The overall length is about 1.8 cm in total as shown in Figure 4A for an adult mouse.

⚠ CRITICAL: Mouse should be euthanized in a fume hood to avoid allergies caused by animal hairs and taken out after skin is removed. Dissection has to be done as soon as possible after the mouse has been sacrificed. Keep the cell culture dishes on ice the whole time. Sciatic DRG must be harvested before nerve dissection for the nerves to be easily pulled out.

Dorsal root dissection

⏲ Timing: ~ 4 h

This section describes the steps of the sciatic dorsal root dissection 24 h after spinal cord injury.

6. When collecting sciatic dorsal roots from mice that received a spinal cord injury or laminectomy, fix them on the foam board with their backs down.
7. Dissect the tissues under the dissection microscope.
   a. Cut the ventral vertebrae using the student vannas spring scissors carefully without cutting the dorsal roots and expose the spinal cord with forceps.
   b. Carefully dissect L4-L6 sciatic DRG out from the intervertebral foramen using forceps without breaking the roots.
   c. Separate the dorsal from the ventral root and track along the spine until you reach the dorsal root entry zone.
   d. Collect the dorsal root segment that extends from the DRG to the dorsal root entry zone. The L6 dorsal root is the longest (~ 1.5cm) as shown in Figure 3B.
   e. Pool dorsal roots from 7 and 8 mice in one 3.5cm cell culture dish and always keep the dish on ice. Prepare 2 dishes for a total of 15 mice.

⚠ CRITICAL: When sacrificing mice, perform cervical translocation gently without breaking the spinal cord and dorsal roots. Carefully cut and remove the ventral vertebrae as the L6 DRG dorsal root is very thin and easy to be cut off.

Axoplasm extraction

⏲ Timing: ~ 4 h
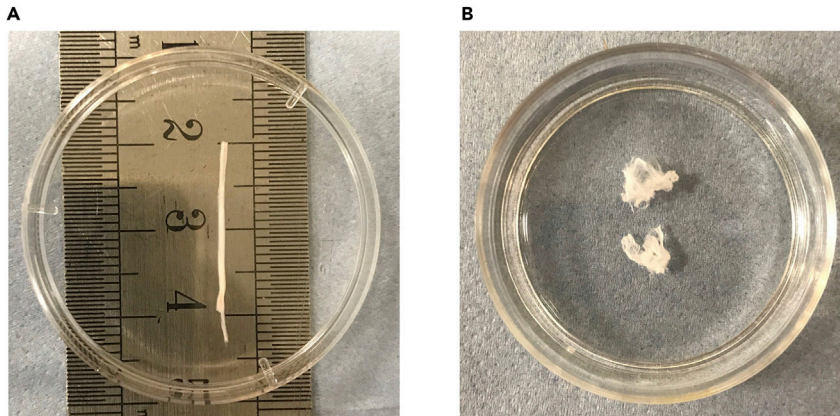
Figure 4. Sciatic nerve length measurement and fascicles separation
(A and B) Measuring sciatic nerve segment length (A) and separation of fascicles (B)

This section explains how to extract axoplasm after tissue dissection.

8. Transfer the nerves to a new 3.5 cm cell culture dish with 2 mL 0.2× PBS with protease and phosphatase inhibitors.
9. Cut each nerve into 2 pieces. Gently expose and separate nerve fascicles under the dissection microscope with fine forceps until they become cloudy and float in the dish as shown in Figure 4B.
10. Transfer the floating fascicles to a fresh 2 mL Eppendorf tube containing 500 μL 0.2× PBS with inhibitors, keep the tube on ice. Transfer fascicles immediately after they become cloudy and start the separation on the next nerve.
11. Once finish, incubate the Eppendorf tube at 20°C–22°C for 2 h.
12. After incubation, transfer fascicles to a new 2 mL Eppendorf tube and wash them with 1 mL 0.2× PBS with inhibitors. Wash 5 min on the roller mixer.
13. Repeat the washing steps 2 more times.
14. After washing, transfer the fascicles into a new empty 2 mL Eppendorf tube to remove liquid, repeat this step once.
15. Transfer the fascicles to a new 1.5 mL Eppendorf tube containing 300 μL 1× PBS with protease and phosphatase inhibitors and incubate at 20°C–22°C for 30 min.
16. Pre-cool the centrifuge to 4°C during incubation, centrifuge at 10,000×g for 10 min at 4°C.
17. Transfer the supernatant to a new 1.5 mL Eppendorf tube, the total volume is about 500 μL.

The axoplasm is ready for concentration.

> ⚠ CRITICAL: Complete the fascicles separation as soon as possible. In the final incubation step (step 15), pool fascicles from the sciatic nerve (10 mice) or from the dorsal roots (15 mice) to one 1.5 mL Eppendorf tube.

> ⧗⧗ Pause point: Stop here for the first day and store the axoplasm at 4°C for up to 72 h or proceed to the next concentration step.

Axoplasm concentration and buffer exchange

> ⏱ Timing: ~ 4 h

This section describes the steps of axoplasm concentration and buffer exchange before mass spectrometry.

18. Prepare the Amicon® Pro device by attaching the Amicon® Ultra-0.5 filter to the base of the exchange device.
19. Add 500 μL TBST with 1% Tween20 to the exchange device, close the cap and centrifuge at 4,000×*g* for 1 min at 4°C.
20. Add 500 μL 1× PBS with inhibitors to the exchange device, close the cap and centrifuge at 4,000×*g* for 1 min at 4°C.
21. Remove the Amicon® Ultra-0.5 filter and place a 2 mL Eppendorf tube over top and invert. Centrifuge at 1,000×*g* for 2 min at 4°C.
22. Add purified axoplasm to the exchange device and centrifuge 4,000×*g* for 30 min at 4°C in a swinging bucket rotor.
23. After sample concentration, add 500 μL denaturation buffer to the exchange device. Centrifuge at 4,000×*g* for 2 h at 4°C. The final volume should be about 30 μL.
24. Remove the Amicon® Ultra-0.5 filter and place a collection tube which is provided in the Purification System over the top and invert. Centrifuge at 1,000×*g* for 2 min at 4°C.
25. Store the concentrated samples at −80°C for the Mass Spectrometry analysis.

> △ CRITICAL: In step 23, after 2 h centrifugation, check sample volume, if the volume is more than 30 μL, increase the centrifuge time until the final volume is about 30 μL. In step 24, when removing the Amicon® Ultra-0.5 filter from the exchange device, depress the cap at the same time to expel the remaining sample that is held by the exchange device tip into the filter.

> ⏸ **Pause point:** Stop here. Proceed to the mass spectrometry experiment after collecting the axoplasm samples from all the groups.

Mass spectrometry sample preparation

> ⏱ Timing: ~ 1 day

This section describes the steps of axoplasm protein sample preparation for mass spectrometry.

26. Heat the axoplasm samples at 70°C for 10 min in 1× NuPAGE LDS Sample Buffer with 100 mM DTT. 20 ug protein is loaded per sample.
27. After, separate proteins on 10% NuPAGE Bis-Tris gel in MES running buffer for 10 min at 180 V.
28. Fix the gel in 7% acetic acid containing 40% methanol for 15 min.
29. Stain the gel using the Colloidal Blue Staining Kit for 30 min.
30. After staining, cut the lanes containing proteins from the gel.
31. Chop the gel slices and collect them in a 1.5 mL Eppendorf tube. Incubate with 1 mL 50% ethanol in 25 mM NH$_4$HCO$_3$ at 20°C–22°C with rotating followed by several rounds of exchange until destained.
32. Remove destain buffer, dehydrate the gel pieces in 100% acetonitrile for 10 min with rotating until they are white and compact.
33. Remove the supernatant, dry sample gel pieces with the vacuum (using Vacuum centrifuge).
34. Rehydrate and reduce the vacuum-dried samples in 150 μL reduction buffer (10 mM DTT in 50 mM NH$_4$HCO$_3$, pH 8.0) for 60 min at 56°C.
35. Remove buffer, add 150 μL 50 mM iodoacetamide in 50 mM NH$_4$HCO$_3$, pH 8.0 and incubate at dark for 45 min at 20°C–22°C.
36. Wash gel pieces once in 50 mM triethylammonium bicarbonate buffer, pH 8.0.
37. Remove the solution and repeat steps 32 and 33, then add 160 μL trypsin digestion buffer (1 μg trypsin per sample in 50 mM triethylammonium bicarbonate buffer, pH 8.0) and incubate 1–12 h at 37°C.

> △ CRITICAL: Remove all acetonitrile before trypsinization to ensure the complete digestion of proteins.

Peptides extraction, purification, and desalting

⏱ Timing: ∼ 1 day

Peptides are extracted, purified, and desalted in this section.

38. Collect the supernatant which contains the protein peptides to a new Eppendorf tube.
39. Add 100 μL 30% acetonitrile extraction solution to the gel pieces and incubate for 15 min at 20°C–22°C with shaking at 1,400 rpm.
40. Collect the supernatant and combine it with the solution obtained in step 38.
41. Repeat steps 39 and 40.
42. Add 100 μL 100% acetonitrile and incubate at 20°C–22°C for 15 min with sharking at 1,400 rpm.
43. Collect the supernatant and combine it with the previous supernatant.
44. Repeat steps 42 and 43.
45. Reduce supernatant volume with vacuum to remove acetonitrile until ∼100 μL is left.
46. Perform reductive methylation labeling for quantification as described previously (Hsu et al., 2003).
47. After labeling, purify peptides using C18 StageTips as described in (Rappsilber et al., 2007).
    a. Activate and equilibrate C18 material with 50 μL each 100% methanol, 80% acetonitrile with 0.1% formic acid and 0.1% formic acid before loading peptides.
    b. Load peptides supernatant.
    c. Wash with 0.1% formic acid.
    d. Elute the peptides in 30 μL 80% acetonitrile with 0.1% formic acid and dry it with vacuum.
48. Reconstitute peptides in 14 μL of 0.1% formic acid.
49. Load 3.5 μL peptides for liquid chromatography-tandem mass spectrometry analyses. For detailed parameters of HPLC and mass spectrometer used for this study, please refer to our publication (Kong et al., 2020).

⚠ CRITICAL: Reductive methylation labeling can only be performed in the absence of ammonium ions which is given when using triethylammonium bicarbonate. It also provides buffering conditions to restrict the labeling reaction in a pH range of 5-8.5.

Mass spectrometry analyses

⏱ Timing: ∼ 1 day

This section describes the steps of mass spectrometry analysis.

50. For peptides identification and protein quantification, perform a database search using MaxQuant Version 1.5.2.8 (Cox and Mann, 2008) against the *Mus musculus* Ensembl database (release-81; 3 July 2015; 53,819 entries).
51. Apply the following settings and thresholds:
    a. Variable modification: acetyl (protein N-term), oxidation (M).
    b. Fixed modifications: carbamidomethyl (C).
    c. Apply FDR < 0.1 for the peptides and protein analyses.
    d. Set dimethylLys0 and dimethylNter0 as light labels and dimethylLys4 and dimethylNter4 as heavy labels, these are set with a maximum of three labeled amino acids.
    e. Proteins with at least two unique peptides are considered as identified.
    f. Filter out proteins identified only with peptides containing a modification.
    g. Apply MaxQuant default settings for protein quantification.
    h. Calculate the ratio between heavy and light using two unique peptides per protein.
    i. Convert proteins' Ensembl ID to gene names using the 'biomaRt' package.
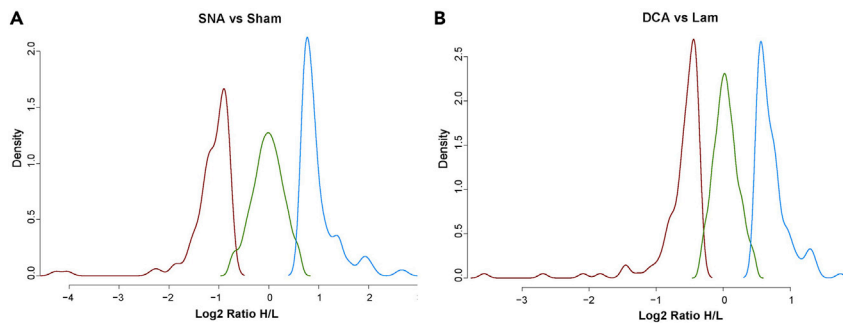
Figure 5. Protein distributions using deconvolution statistical analysis after SNA and DCA
(A and B) Histograms show the protein density distribution of SNA vs Sham (A) and DCA vs Lam (B). Probability density function showing the distribution of log2 heavy/light value. The noise distribution (green) is centered around 0 and the two subpopulations on either tail of the overall distribution are shown (blue= ratio>0 and red=ratio <0).

52. Apply deconvolution statistical approaches to calculate the differential protein expression between injury and sham conditions. The pipeline was described in our publication (Kong et al., 2020).

   ⚠ CRITICAL: Ensure all settings are correct and the correct database is loaded.

EXPECTED OUTCOMES

In our study, we identified 3128 protein groups across all replicates in these four conditions; the whole protein list was provided in our publication (Kong et al., 2020). In the differential protein expression analysis, we assume that the dimethyl signal can be modeled as the combination of technical noise and actual protein level. The background noise is normally distributed and centered on zero, while the biological response to stimuli (injury in this case) is normally distributed along the extreme edges of the overall distribution. Thus, we expect three normal distributions, one in the center and one on each side as shown in Figure 5. Proteins that are only detected in one replicate are excluded. After performing a one-tailed *t*-test with Bonferroni-Hochberg multiple corrections (FDR) of each detected protein relative to the null hypothesis, proteins are future filtered with an additional cut-off absolute log2 Ratio > 0.58. Red dots in Figure 6 are significantly down or upregulated proteins following SNA or DCA versus their own sham control. Finally, 172 and 140 differentially expressed proteins are found following SNA or DCA respectively. The codes used for the analyses in Figures 5 and 6 are available in the link provided in the data and code availability section.

LIMITATIONS

Although we successfully discovered numerous differentially expressed proteins in axoplasm from both sciatic nerve and dorsal roots following sciatic nerve or spinal cord injury, due to the limited material obtained from mice, it remains challenging to this day to investigate post-translational modifications from axoplasmic proteins such as phosphorylation or acetylation, especially from the more scarce material obtained from the dorsal roots. Development and refinement of mass spectrometry methodologies will hopefully be able to reduce the required amount of material and allow for the study of post-translational modifications. Additionally, the possibility of having a lower starting material for proteomics will address another limitation that is the high number of mice currently needed for each biological replicate. It must also be stated that axoplasmic protein extraction represents a substantial enrichment for axonal proteins but that a small fraction of extra neuronal proteins can still be found in the axoplasmic preparation. Lastly, investigation of axonal trafficking between the neuron cell body and the synapse or injury site cannot be addressed by performing axoplasm proteomics alone.
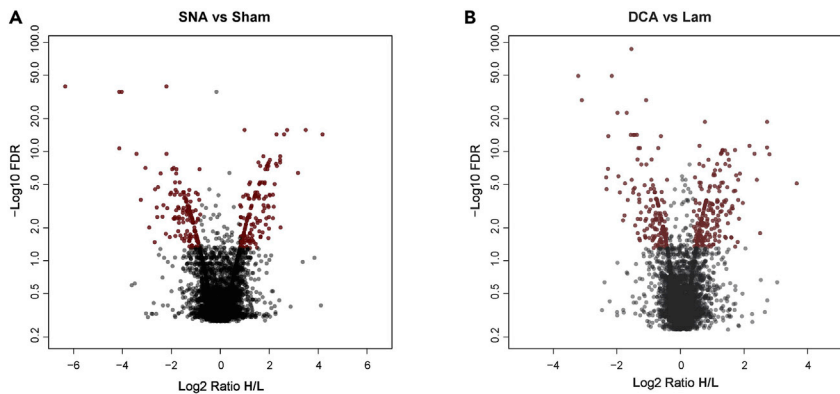
Figure 6. Differentially expressed protein analysis following SNA and DCA
(A and B) Volcano plots show the differentially expressed proteins of SNA vs Sham (A) and DCA vs Lam (B).
Proteins marked in red are the significantly down or upregulated proteins after SNA or DCA. FDR< 0.05, log2
ratio> 0.58 or < −0.58.

TROUBLESHOOTING

Problem 1

Spinal cord and dorsal roots are broken before dorsal root dissection (step 7), making the dissection
more difficult and even causing some dorsal roots material loss.

Potential solution

When sacrificing mice, gently perform cervical translocation and remove the skin and internal
organs.

Problem 2

Axoplasm concentration takes a very long time after adding a denaturation buffer (step 23).

Potential solution

Check whether there is residual tissue attached to the filter membrane. Make sure the pipette tip
doesn't touch tissues on the tube bottom when collecting axoplasm supernatant after centrifugation
in step 17.

Problem 3

Occasionally, a lower protein amount (< 20 ug) is obtained from dorsal roots that are collected from
15 mice (step 26). This is typically due to dorsal root loss during dissection.

Potential solution

To avoid the risk of having dorsal roots axoplasm enrichment below 20 ug, make sure that all dorsal
roots have been dissected and collected, especially the L6 dorsal roots, which are very thin and
harder to dissect.

Problem 4

In some samples, Schwann cell proteins such as S100 and GFAP can be found in the enriched
axoplasm. See the identified proteins list provided in our publication (Kong et al., 2020).

Potential solution

Gently expose and separate fascicles to avoid excessive Schwann cell damage.

Problem 5

Trypsin digestion efficiency is not sufficient (step 37). After mass spectrometry analysis perform database search (mus musculus) allowing 0, 1, and 2 missed cleavages of trypsin. The majority of peptides (> 80 %) should have no missed cleavage while some have one missed cleavage and very little (< 2%) have two missed cleavages. If the cleavage efficiency drops suddenly, the trypsin digestion step has to be troubleshot.

Potential solution

Check if the commercially available trypsin is still active by digesting a protein standard: e.g., purified bovine serum albumin (Bovine Serum Albumin, Roche, #10711454001). Check if the digestion buffer is compatible with commercially available trypsin and does not contain any activity suppressing components. Assure that gel pieces have been washed in digestion buffer (50mM triethylammonium buffer), dehydrated with 100% acetonitrile, and dried with vacuum before trypsin digestion. Check if time and temperature during trypsin digestion are appropriate.

Problem 6

Reductive methylation is insufficient (step 46).

Potential solution

Make sure that (i) the trypsin digestion buffer contains no primary amines; (ii) extracted peptides from gel pieces are free of acetonitrile by evaporation; (iii) sufficient label is provided during labeling reaction; (iv) the correct pH of 5–8.5 has been used.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Simone Di Giovanni (s.di-giovanni@imperial.ac.uk).

Materials availability

This study did not generate new unique reagents.

Data and code availability

Raw axoplasm proteomics data used for the analyses are available in ProteomeXchange Consortium: PXD013297 and supplementary table 1 in our publication (Kong et al., 2020). Codes used to create histograms in Figure 5 are provided in our publication (Kong et al., 2020). Codes used to create the volcano plots in Figure 6 are available at https://github.com/gitgpk/Star-Protocols.git.

AUTHOR CONTRIBUTIONS

G.K. performed the experiments and data analysis and wrote the paper; L.Z. performed the experiments; A.F. performed the mass spectrometry experiments and data analysis; K.S. performed data analysis; S.D.G. designed experiments, provided funding, and edited the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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