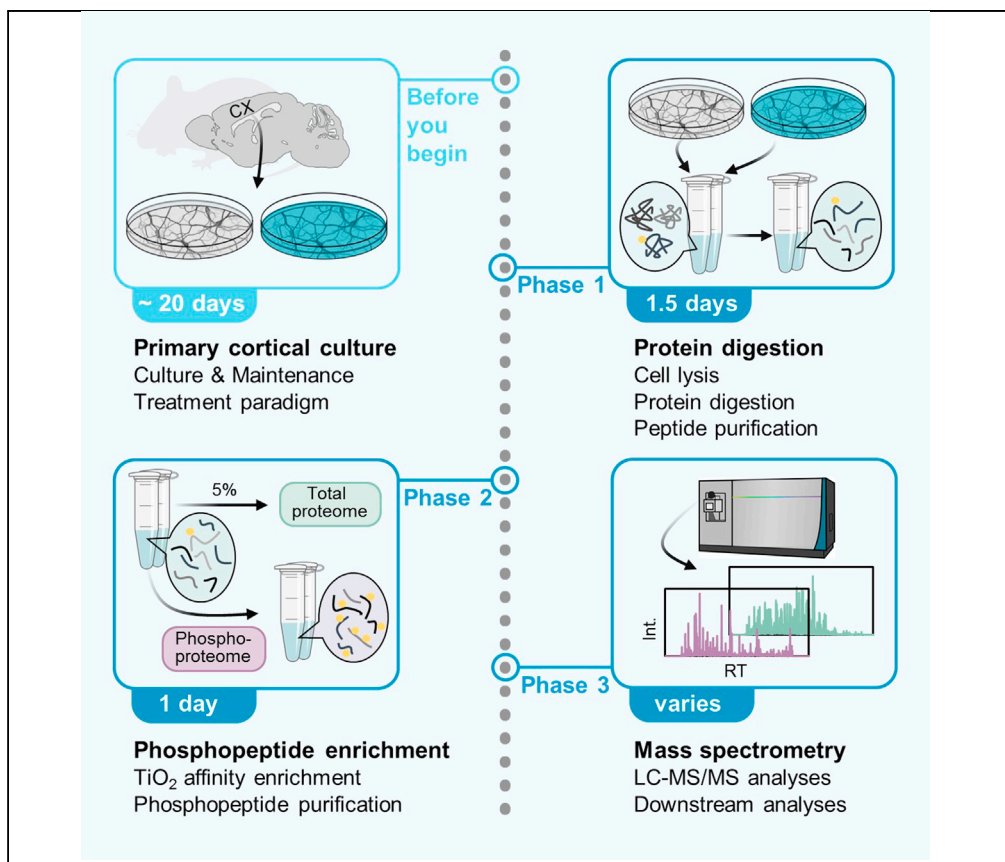


Protocol

Quantifying phosphorylation dynamics in primary neuronal cultures using LC-MS/MS



Cellular processes require tight and coordinated control of protein abundance, localization, and activity. One of the core mechanisms to achieve specific regulation of proteins is protein phosphorylation. Here we present a workflow to monitor protein abundance and phosphorylation in primary cultured neurons using liquid chromatography-coupled mass spectrometry. Our protocol provides a detailed guide on all steps for detection and label-free-quantification of phosphorylated and unmodified proteins of primary cortical neurons, including primary cell culture, phosphoproteomic sample preparation and data-processing, and evaluation.

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Highlights

Preparation and maintenance of rat primary-cultured neuronal cells

Detailed protocol on phosphoproteomics sample preparation from cortical neurons

Core steps of downstream (phospho-) proteomics data processing and statistical analyses

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Protocol

Quantifying phosphorylation dynamics in primary neuronal cultures using LC-MS/MS

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SUMMARY

Cellular processes require tight and coordinated control of protein abundance, localization, and activity. One of the core mechanisms to achieve specific regulation of proteins is protein phosphorylation. Here we present a workflow to monitor protein abundance and phosphorylation in primary cultured neurons using liquid chromatography-coupled mass spectrometry. Our protocol provides a detailed guide on all steps for detection and label-free-quantification of phosphorylated and unmodified proteins of primary cortical neurons, including primary cell culture, phosphoproteomic sample preparation and data-processing, and evaluation.

For complete details on the use and execution of this protocol, please refer to Desch et al. (2021).

BEFORE YOU BEGIN

Primary neuronal cultures are an excellent model system that enables pharmacological and electrophysiological manipulation and analysis of neuronal cells. Primary cultured neurons exhibit physiological behavior, including polarization, the formation of axonal and dendritic processes as well as the generation of fully functional synaptic connections. Here, dissociated cortical neurons are prepared from Sprague Dawley rat pups as previously reported (Aakalu et al., 2001). To support neuronal development and to keep neurons in near-physiological conditions for biochemical or imaging experiments, the cells require external trophic support when kept in culture. Our strategy involves the use of pre-conditioned medium to support the survival of the plated neurons (Figure 1). For this purpose, we typically prepare “feeder cultures” of cortical and glia cells before the experiment. Over a period of 40 days, we harvest and collect conditioned medium containing a variety of secreted factors for later use, as described in the following sections.

All experiments explained in the following section complied with national animal care guidelines, the guidelines issued by the Max Planck Society and were approved by local authorities.

Poly-D-lysine coating of petri dishes and flasks

⌚ Timing: 9–16 h

1. Add 5 mL of poly-D-lysine solution onto 10 cm petri dishes or 60 mL onto cell culture flasks (3-layered). Work under a biosafety hood in sterile conditions.
2. Keep the dishes or flasks in an incubator at 37°C overnight or for at least 8 h.



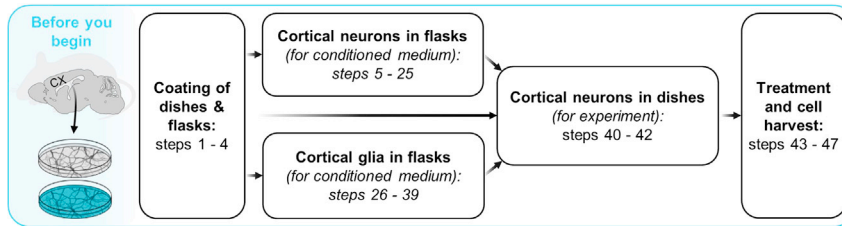


Figure 1. “Before you begin” overview: workflow for primary cortical cell culture

Boxes indicate all procedures to set up and maintain primary cultures of cortical neurons, with details for each step listed below. Before neurons for the actual experiment are plated and manipulated, cell culture dishes require poly-D-lysine coating. In addition, conditioned medium from two different “feeder” cultures needs to be harvested.

3. Remove the solution and wash with sterile water. Repeat this step twice, for a total of three washes.
4. Remove the solution and keep the dishes or flasks in the biosafety hood until they are completely dry and ready to use.

Note: The number of dishes required for the experiment depends on its design. Typically, one dish is plated with 3 million cells which yields sufficient protein to study one experimental condition (>350 µg protein for digestion and enrichment). The cortex preparation of one rat (P0 or P1) yields approximately 15–20 million cortical cells. More than a single litter can be required.

⏸ **Pause point:** Coated dishes can be prepared beforehand, wrapped in aluminum foil and stored at 4°C for approximately 1 week.

Preparation of cortical neurons in flasks

⌚ **Timing:** 40 d

5. Sacrifice postnatal rat pups (P0 to P1) using an approved method of euthanasia (expected yield: 15–20 mio. cells/ animal).
6. Decapitate the animals and dissect the brains.
7. Place the brains into a petri dish containing some ice-cold dissociation medium (DM), ensuring the tissue is in contact with the liquid and does not dry out.
8. Remove the cerebellum and split the hemispheres. Remove the midbrain and meninges to separate the cortices.
9. Transfer and collect all cortices in a 50 mL centrifugation tube in 5 mL DM on ice.
10. Cut the cortices into smaller pieces using a sterile scalpel or spatula.
11. Gently remove the DM.
12. Add 5 mL of warm cysteine-papain solution to the tissue and incubate for 15 min at 37°C in a water bath.
13. Remove the supernatant and incubate with fresh cysteine-papain solution for additional 15 min at 37°C.
14. Remove the supernatant and gently wash the cells with 10 mL ice-cold DM to stop digestion. Repeat this step 4–7 times.
15. Remove the supernatant and wash with 10 mL ice-cold neuronal growth medium (NGM). Repeat this step once.
16. Remove the supernatant and triturate the cortices in 8 mL ice-cold NGM by pipetting up and down 5–10 times using a serological pipette (10 mL).
17. Incubate on ice for 3 min to allow non-dispersed tissue to settle from the single cell suspension.
18. Take the supernatant into a new 50 mL centrifugation tube.
19. Centrifuge for 5 min at 67 × g at 4°C. Discard the supernatant.

20. Resuspend the pellet in 10 mL NGM.
21. Keep the cells on ice until plating and determine cell count of the suspension using a Neubauer chamber.
22. Plate 15 mio. cells per poly-D-lysine-coated flask (3-layered) in approximately 60 mL volume. Keep the flasks at 37°C and 5% CO₂.
23. Change medium to fresh NGM 4 h after plating.
24. Change medium after 3–4 days to fresh NGM. Start to collect the conditioned supernatant.
25. Change medium in bi-weekly schedule (i.e., Monday and Friday) and harvest conditioned medium for approximately 40 days. Pool the harvested medium and store the conditioned “cortical medium” at –20°C.

Note: Buffer volumes noted in each steps depend on the number of animals used in the preparation as well as the experience and efficiency in dissection and/or cell trituration/ preparation. Increasing or decreasing of buffer volumes can be necessary, especially when resuspending the cell pellets before plating (step 20).

Note: For additional, visual guidance and to make the preparation more accessible to beginners as well, we refer to the multimedia presentation by [Viesselmann et al. \(2011\)](#).

Preparation of cortical glia in flasks

⌚ Timing: 40 days

26. Perform step 5–13.
27. Remove the supernatant and gently wash the cells with 10 mL ice-cold DM supplemented with 12.5% FCS to stop digestion. Repeat this step once.
28. Remove the supernatant and gently wash the cells with 10 mL ice-cold DM to stop digestion. Repeat this step three times.
29. Remove the supernatant and gently wash the cells with 10 mL ice-cold minimum essential medium (MEM).
30. Remove the supernatant and triturate the cortices in 8 mL cold MEM by pipetting up and down 5–10 times using a serological pipette.
31. Incubate on ice for 3 min to allow non-dispersed tissue to settle from the single cell suspension.
32. Take the supernatant into a new 50 mL centrifugation tube.
33. Centrifuge for 5 min at 67 × g at 4°C. Discard the supernatant.
34. Resuspend the pellet in MEM (15 mL per flask; pellet of 6–10 animals in approx. 45 mL).
35. Prepare 45 mL MEM per poly-D-lysine-coated flask (3-layered) and add 15 mL cell suspension for plating. Keep the flasks at 37°C and 5% CO₂.
36. Change medium to fresh MEM 4 h after plating.
37. Change medium to fresh MEM 3–4 days after plating.
38. Change medium to fresh NGM 7 days after plating.
39. Change medium in bi-weekly schedule (i.e., Monday and Friday) and harvest the conditioned NGM-medium for approximately 40 days. Pool the harvested medium and store the conditioned “glia medium” at –20°C.

Note: Similar to the previous section, buffer volumes noted in each step might require adaptations depending on the number of animals used and the preparative skills, in particular during cell resuspension (step 34).

Preparation of cortical neurons in petri dishes

⌚ Timing: 19–20 days

40. Repeat step 5–21.
41. Plate cortical neurons at a density of 3 mio. cells per 10 cm poly-D-lysine-coated petri dish.
42. Keep the cultures in 5 mL (per 10 cm petri dish) NGM at 37°C and 5% CO₂ for over 18 days to ensure synapse maturation.
 - a. Feed the cells by adding 2.5 mL of conditioned medium – a sterile-filtered mix of 80% fresh NGM, 15% conditioned “cortical medium” (step 25) and 5% conditioned “glia medium” (step 39) - in a weekly schedule.

Cell treatment and harvest

⌚ Timing: 1 h, depending on treatment

In a previous study, we used this protocol to investigate phosphoregulation during homeostatic synaptic scaling in neurons (Desch et al., 2021). To induce homeostatic scaling, we added the pharmacological reagents bicuculline (20 μM) or tetrodotoxin (1 μM) to the culture medium. Bicuculline functions as a GABA-receptor antagonist blocking action potentials of inhibitory neurons and inducing synaptic down-scaling (O’Brien et al., 1998). Tetrodotoxin acts as a sodium-channel blocker leading to a cessation of action potentials and ultimately to up-scaling (Turrigiano et al., 1998). Here we describe the general steps of the treatment and harvest procedure keeping the treatment paradigm - with concentration and duration of stimulation – optional for the experimenter.

43. After 19–20 days, add the pharmacological treatment of choice to the medium. Work under a biosafety hood in sterile conditions.
 - a. Remove half of the medium and transfer it to a fresh 15 mL centrifugation tube. Make sure that the cells in the dish are still covered.
 - b. Mix the medium with the drug according to the final concentration.
 - c. Gently re-add the drug-containing medium to the cells.
 - d. Incubate at 37°C and 5% CO₂. Choose durations according to your treatment paradigm.
44. Remove the cell culture medium.
45. Wash the cells with 3 mL ice-cold DPBS supplemented with protease and phosphatase inhibitor. Repeat this step once.
46. Scrape the cells in 0.75 mL ice-cold PBS supplemented with protease and phosphatase inhibitor and transfer the suspension in a 2 mL reaction tube. Repeat this step once.
47. Centrifuge at 2,000 × g for 8 min at 4°C. Discard the supernatant.

⏸ Pause point: Cell pellets can be snap-frozen in liquid nitrogen and stored at –80°C for at least a year.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Poly-D-lysine	Sigma-Aldrich	Cat# A003E
Papain	Sigma-Aldrich	Cat# P3125
L-cysteine	Carl Roth	Cat# 1693.1
Neurobasal-A	Life Technologies	Cat# 10888022
B27	GIBCO	Cat# 17504044
GlutaMax	GIBCO	Cat# 35050038
Fetal Bovine Serum (FBS/ FCS)	Invitrogen	Cat# 16000044
MEM	Invitrogen	Cat# 310985
Glucose	Sigma-Aldrich	Cat# G7528

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Horse Serum	Sigma-Aldrich	Cat# H1138
DPBS (1x)	GIBCO	Cat# 14190094
PhosStop phosphatase inhibitor	Roche, Sigma-Aldrich	Cat# 4906845001
cOmplete protease inhibitor cocktail (EDTA-free)	Roche, Sigma-Aldrich	Cat# 4693159001
Benzonase	Sigma-Aldrich	Cat# E1014
SDS (20%)	AppliChem	Cat# A0675
TRIS	Sigma-Aldrich	Cat# 252859
Iodoacetamide (IAA)	Thermo Fisher Scientific	Cat# A39271
Dithiothreitol (DTT)	Sigma-Aldrich	Cat# D5545
Ammoniumbicarbonate (ABC)	Sigma-Aldrich	Cat# 09830
LC-MS grade methanol	Carl Roth	Cat# AE71.1
Sequencing grade modified trypsin	Promega	Cat# V5111
LC-MS grade acetonitrile (ACN)	Carl Roth	Cat# AE70.1
LC-MS grade water	Riedel-de Haën/ Honeywell	Cat# 39253
Formic acid (FA)	Fluka/Honeywell	Cat# 94318
Trifluoroacetic acid (TFA)	Carl Roth	Cat# P088.1
Acetic acid (glacial)	Sigma-Aldrich	Cat# A6283
Bicuculline methochloride	Tocris Bioscience	Cat# 0131
Tetrodotoxin citrate	Tocris Bioscience	Cat# 1069
Critical commercial assays		
BCA protein assay kit	Thermo Fisher Scientific	Cat# 23225
TiO ₂ Phosphopeptide Enrichment Kit	Thermo Fisher Scientific	Cat# A32993
Deposited data		
Phosphoproteomics data (homeostatic scaling)	Desch et al. (2021)	PRIDE: PXD021834
Experimental models: Cell lines		
Rat cortical cultured neurons from P0-1	Charles Rivers	RRID: RGD_734476
Experimental models: Organisms/strains		
Rattus norvegicus, Sprague-Dawley	Charles Rivers	RRID: RGD_734476
Software and algorithms		
MaxQuant (1.6.6.0)	Cox and Mann (2008)	RRID:SCR_014485
UniprotKB	www.uniprot.org/uniprot/	RRID:SCR_004426
Perseus (1.6.2.3)	Tyanova et al. (2016)	RRID:SCR_015753
R for statistical computing	n/a	www.r-project.org/
Other		
3-layered flask (TripleFlasks)	Nunc, VWR	Cat# 7342001
10 cm petri dishes (Cellstar)	Greiner Bio-One, Sigma-Aldrich	Cat# P7612
Sterile filter (Filtropur S 0.2)	Sarstedt	Cat# 83.1826.001
S-Trap (mini columns)	ProtiFi	protifi.com/pages/s-trap
Sep-Pak cartridge C ₁₈ (100 mg sorbent)	Waters	Cat# WAT023590
Empore 3M C ₁₈ membrane	Supelco	Cat# 66883-U
C ₁₈ trapping column	Thermo Scientific	Cat# 164535
C ₁₈ analytical column (50 cm)	CoAnn Technologies, LLC	Cat# HEB07505001718IWF
QuanRecovery autosampler vials	Waters	Cat# 186009186

MATERIALS AND EQUIPMENT

Poly-D-lysine solution

Reagent	Final concentration	Stock concentration	Volume
Poly-D-lysine	0.1 mg/mL	1 mg/mL	25 mL
MiliQ H ₂ O	n/a	n/a	225 mL

This solution must be sterile-filtered (0.2 μm pore size; as for all further filtrations) and stored at 4°C.

Dissociation medium (DM)

Reagent	Final concentration	Stock concentration	Volume
Na ₂ SO ₄	82 mM	1 M	41 mL
K ₂ SO ₄	30 mM	0.5 M	30 mL
MgCl ₂	5.8 mM	1 M	2.9 mL
CaCl ₂	0.252 mM	1 M	0.126 mL
HEPES	1 mM	1 M	0.5 mL
Glucose	20 mM	2.5 M	4 mL
Phenolred	0.001% (w/v)	n/a	5 mg
MiliQ H ₂ O	n/a	n/a	421.474 mL

This solution must be sterile-filtered and stored at 4°C.

Cysteine-papain-solution

Reagent	Final concentration	Stock concentration	Volume
L-cysteine	0.32 mg/ mL	n/a	6.4 mg
Papain solution	n/a	n/a	0.6 mL*
NaOH	1 mM	1 M	20 µL*
DM	n/a	n/a	20 mL

This solution must be sterile-filtered and stored at 4°C. It is stable for approximately 1 h.

*Add immediately before use; solution should have a slightly pink color.

Neuronal growth medium (NGM)

Reagent	Final concentration	Stock concentration	Volume
B27	1×	50×	20 mL
GlutaMax	1×	100×	10 mL
Neurobasal-A	n/a	n/a	970 mL

This solution must be sterile-filtered and stored at 4°C.

Minimum essential medium (MEM)

Reagent	Final concentration	Stock concentration	Volume
Horse serum	n/a	n/a	50 mL
Glucose	0.4%	20%	10 mL
MEM	n/a	n/a	440 mL

This solution must be sterile-filtered and stored at 4°C.

Lysis buffer (2×)

Reagent	Final concentration	Stock concentration	Volume
TRIS-HCl pH 7.5	100 mM	1 M	1 mL
SDS	10% (v/v)	20%	5 mL
Protease inhibitor	2×	50×	*
Phosphatase inhibitor	2×	25×	*
MiliQ H ₂ O	n/a	n/a	4 mL

This solution can be stored at room temperature (20°C–22°C) up to several months; evaluation of pH is recommended.

*Add protease and phosphatase inhibitors freshly before use.

Protein binding buffer

Reagent	Final concentration	Stock concentration	Volume
TRIS pH 7.1 (adj. with H ₃ PO ₄)	50 mM	0.5 M	10 mL
Methanol	90%	n/a	90 mL

This solution needs to be freshly prepared and can be kept at room temperature (20°C–22°C) for several hours.

Digestion buffer

Reagent	Final concentration	Stock concentration	Volume
Ammoniumbicarbonate	50 mM	n/a	40 mg
MiliQ H ₂ O	n/a	n/a	10 mL

This solution needs to be freshly prepared. Right before digestion, trypsin is added into the buffer to generate a master mix containing the protease according to the enzyme-to-protein ratio.

Reconstitution buffer

Reagent	Final concentration	Stock concentration	Volume
Formic acid	0.1%	100%	1 μ L
MS-grade acetonitrile	2%	n/a	20 μ L
MS-grade H ₂ O	98%	n/a	979 μ L

LC-solvent: Buffer A

Reagent	Final concentration	Stock concentration	Volume
Formic acid	0.1%	100%	0.25 mL
MS-grade H ₂ O	n/a	n/a	249.75 mL

This solution needs to be degassed in a water bath sonicator for 15 min before use and can be stored for 1–3 months.

LC-solvent: Buffer B

Reagent	Final concentration	Stock concentration	Volume
Formic acid	0.1%	100%	0.25 mL
MS-grade acetonitrile	80%	n/a	200 mL
MS-grade H ₂ O	20%	n/a	49.75 mL

This solution needs to be degassed in a water bath sonicator for 15 min before use and can be stored for 1–3 months.

LC-solvent: loading buffer

Reagent	Final concentration	Stock concentration	Volume
Trifluoroacetic acid	0.05%	100%	0.25 mL
MS-grade acetonitrile	2%	n/a	10 mL
MS-grade H ₂ O	98%	n/a	498.75 mL

This solution needs to be degassed in a water bath sonicator for 15 min before use and can be stored for 1–3 months.

nano-HPLC gradient (phosphoproteome profiling)

Time	LC-solvent B [%]	Flow [nL/min]
0	4	300
6	4	300
116	30	300
126	45	300
127	90	300
132	90	300
133	4	300
153	4	300

nano-HPLC gradient (total proteome profiling)

Time	LC-solvent B [%]	Flow [nL/min]
0	4	300
6	4	300
186	48	300
187	90	300
192	90	300
193	4	300
213	4	300

Orbitrap Fusion Lumos method

Parameter	Value
Polarity	Positive
Internal calibration (lock-mass)	445.12003 m/z
Full scan – MS1	
Orbitrap resolution	120k at 200 m/z
Scan range	350–1400 m/z
AGC target	2e5
Maximum injection time	50 ms
dd scan – MS2	
Data-dependent mode	Cycle time
Time between master scans	2 s
Quadrupole isolation window	1.4 m/z
HCD collision energy	30%
Ion trap scan rate	Rapid
AGC target	1.5e4
Maximum injection time	300 ms
Dynamic exclusion	30 s
Charge inclusion	2–5
Inject ions for all available parallelizable time	true

STEP-BY-STEP METHOD DETAILS

This guide is designed to enable identification and quantification of individual phosphorylation events and proteins by LC-MS. All necessary phases of the experiments are explained in detailed steps (for an overview, see [Figure 2](#)), including lysis of the cells, bottom-up proteomic sample preparation, LC-MS data acquisition and raw-data processing and analyses. The LC-MS analysis and respective down-stream processing are carried out for samples of the unenriched (total proteome) and phospho-enriched peptide samples (phosphoproteome).

Cell lysis

⌚ Timing: 1–2 h

The first step of the protocol aims at physical cell disruption to access all cellular contents. To break cells sufficiently, we use the classical approach of mechanical disruption, i.e., a mild method using shearing forces induced by pipetting. To solubilize the proteins – especially hydrophobic membrane proteins – but also to minimize activity of endogenous proteases and phosphatases, we add detergents to the lysis buffer. Homogenous and reproducible cell lysis is essential in proteomics protocols as accurate protein concentration determination as well as digest efficiency heavily rely on this step.

1. Briefly thaw frozen cell pellets in hand.

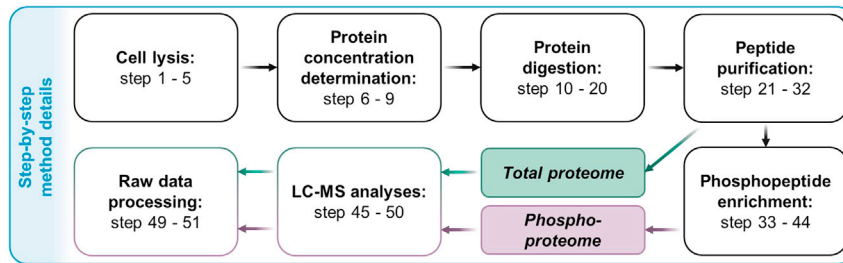


Figure 2. Phosphoproteomics workflow for the quantitative analysis of phosphorylation events in cortical neurons
The major sections of the protocol are shown in the boxes of the flowchart with their corresponding steps listed below. Different coloring of the boxes indicates the two separate analyses for profiling of the phosphoproteome (plum) or the total proteome (turquoise) after splitting the purified peptides of each sample in step 31.

- Lyse the cells in lysis buffer (200 μL per 3 million neurons) by pipetting up and down using a mechanical pipette (i.e., P200). Repeat pipetting at least 15 times or until the solution is clear.
- Add 1 μL benzonase to each sample, mix well by pipetting up and down and incubate for 10 min at room temperature (20°C–22°C).
- Clear the lysate by centrifugation for 10 min at 13,000 $\times g$ at room temperature.
- Transfer the supernatant in a fresh reaction tube.

△ CRITICAL: In step 2, make sure that the cell pellet is lysed homogeneously. If a considerable pellet remains after step 4, lysis was insufficient (troubleshooting 1).

▣ Pause point: Neuronal lysates can be snap-frozen in liquid nitrogen and stored at -80°C for up to a year.

Protein concentration determination

⌚ Timing: 1 h

For comparative, label-free proteomics studies, it is essential to determine the protein concentration in all samples accurately to normalize protein input for each digest preparation. We typically measure protein concentrations using commercially available colorimetric kits such as the Pierce BCA protein quantification kit.

- Prepare a standard curve using bovine serum albumin (BSA; range: 35–2000 $\mu\text{g}/\mu\text{L}$) in lysis buffer (same dilution as samples).
- Perform the BCA-procedure as described in the manufacturer’s protocol.
- Measure the absorbance in a microplate reader at (or around) 562 nm.
- Calculate a standard curve using the values from the different concentrations of BSA and determine the protein concentration of all samples accordingly.

△ CRITICAL: Do not add reducing agents to the sample at this point, as they heavily interfere with the colorimetric assay. Similarly, detergents cause noticeable interference with analysis. Hence, we recommend diluting the sample below 5% SDS for the BCA-analysis.

Note: To generate reliable and precise data, work in technical duplicates and/ or apply different dilutions of each sample.

Note: A protein concentrations of 3–5 mg/mL is within the expected range for the undiluted SDS-lysate. Protein concentrations should be similar for all samples of the same preparation.

Protein digestion

© Timing: 18–20 h

In bottom-up proteomics, proteins are cut into peptides in a controlled digestion reaction. This process is enzymatically catalyzed by proteases that cleave the extracted and solubilized proteins in a residue-specific manner. Trypsin is the most widely-used protease for this type of experiment cleaving the proteins C-terminally of lysine or arginine residues. The cleavage results in peptides with a positively charged amino acid at the C-terminus that is beneficial for the ionization required for LC-MS. Here we use an adapted version of the suspension trapping protocol ([Hailemariam et al., 2018](#); [Zougman et al., 2014](#)) to perform digestion working with commercially available mini spin columns, S-traps (Profiti). We found that in our hands these spin filters are scalable to different sample amounts and generate clean, detergent-free samples with little preparational loss.

Alternatives: This part of the protocol is based on S-trap mini spin columns. This product can be substituted with other centrifugal filter units such as the Microcon centrifugal filter unit with 10 kDa molecular weight cut-off (Millipore). In combination with this type of filter it is, however, necessary to switch to a digestion protocol suitable for the different filter membrane material (as described in [Wiśniewski et al., 2009](#)).

10. Adjust the protein content of all samples to 350 µg protein and prepare them in the same volume. Required volume depends on protein input and/or lysis efficiency. Dilute with lysis buffer if necessary.
11. Add DTT dissolved in lysis buffer to reduce protein disulfide bonds. Use a final concentration of 20 mM.
 - a. Heat and incubate for 5 min at 56°C shaking at 400 rpm.
 - b. Incubate for additional 5 min at room temperature shaking at 400 rpm.
12. Alkylate free sulfhydryl groups on cysteine residues with IAA dissolved in lysis buffer. Use a final concentration of 40 mM.
 - a. Incubate 30 min in the dark shaking with 400 rpm.
13. Add 12% aqueous phosphoric acid at 1:10 for a final concentration of 1.2% acid to the lysate and mix it well.
14. Add six parts of binding buffer to one part of the acidified sample (7:1 ratio) and carefully mix it by pipetting up and down a few times.
15. Add the mix onto the spin column in 400 µL steps. Centrifuge at 4,000 × g for 20 s or until all solvent has passed through the filter. Discard the flow-through when necessary.
16. Wash the captured protein by adding 400 µL protein binding buffer and centrifuge at 4,000 × g for 20 s. Repeat this step for a total of four washes. Discard the flow-through when necessary.
17. Transfer the mini spin column into a new reaction tube.
18. Add 125 µL digestion buffer containing the protease in 1:50 enzyme-to-protein ratio to each sample.
19. Spin down the liquid very briefly (1–2 s) to ensure that the whole filter is soaked in digestion buffer and return any solution that passes to the top of the filter again. Ensure that there are no air bubbles on top of the filter.
20. Incubate the samples overnight (approx. 16 h) in a wet chamber at room temperature. Close the caps of all reaction tubes.

Note: At step 14, the acidified SDS-lysate forms a protein particulate suspension in the organic environment. Do not centrifuge at this point to prevent pellet formation and to ensure a complete transfer of protein to the filter.

Note: Depending on the volume of the lysate, step 15 needs to be repeated several times until all lysate has been loaded onto the column.

Note: Efficiency of the tryptic digest can be checked in the downstream data analysis ([troubleshooting 2](#)).

Note: To prevent evaporative loss during step 20, prepare a wet chamber to generate a humid environment during digestion. Such a chamber can be easily set up using a microtube storage box with a lid and pre-wetted tissue sheets that are placed at the sides within it.

Peptide collection and purification prior phosphopeptide enrichment

⌚ Timing: 1–1.5 h

To remove chemicals that might interfere with the subsequent enrichment of phosphorylated peptides or the following LC-MS analysis of the total proteome, peptides resulting from the digest are washed using C₁₈ cartridges, e.g., the SepPak C₁₈ cartridge with 100 mg sorbent (Waters).

21. Add 80 μ L of digestion buffer (without protease) to the filter unit and centrifuge at 4,000 \times g for 60 s to elute generated peptides.
22. Add 80 μ L of 0.2% FA to the filter unit and centrifuge at 4,000 \times g for 60 s. Repeat this step once.
23. Mix the elution fractions.
24. Condition the Sep Pak C₁₈ cartridge with 2 mL pure ACN. Either apply positive pressure using a syringe to pass the solvent through the resin or employ a vacuum manifold (step 24–30).
25. Wash the sorbent with 1 mL of 50% ACN/ 0.5% acetic acid.
26. Equilibrate the sorbent with 2 mL of 0.1% TFA.
27. Load acidified peptide digests onto the cartridge.
28. Wash and desalt with 1 mL of 0.1% TFA.
29. Wash with 200 μ L 0.5% acetic acid to remove TFA prior elution.
30. Elute the desalted peptides with 200 μ L of 50% acetonitrile/ 0.5% acetic acid into a fresh reaction tube. Repeat this step once.
31. Take a fraction of the sample (5% v/v) and transfer it into a new reaction tube for screening of the total proteome. At this point, there are two samples (95% v/v or 5% v/v) for each experimental and replicate condition.
32. Dry all samples in a speedVac at room temperature.

⚠ CRITICAL: Remember to split the yield of the purified peptides to analyse the proteome (5% v/v of the digest) and the phosphoproteome (95% v/v of digest for phosphopeptide enrichment) before the samples are dried!

Note: If you work with limited sample amounts and/or to maximize the yield, we recommend to repeat step 27 and to re-load the peptides. For this purpose, use a fresh reaction tube before loading and then re-add the solution that passed the tip.

Alternatives: Desalted peptide samples can be dried using a speedVac (step 32) or any other equivalent vacuum dryer, i.e. using a freeze dryer (lyophilisation).

⏸ Pause point: Purified and dried peptides can be snap-frozen in liquid nitrogen and stored at -20°C until LC-analysis or phosphopeptide enrichment for at least 6 months.

Phosphopeptide enrichment and purification

⌚ Timing: 1.5–2 h

Previous in-depth analyses suggest that the majority of (human) proteins may be modified by phosphorylation (around 75%; [Sharma et al., 2014](#)). However, these post-translational modifications are

generally sub-stoichiometric. In complex samples such as neuronal lysates, low-abundant phosphopeptide species are hard to detect as they are masked by unmodified, higher-abundant peptides. Thus, affinity purification or enrichment steps are typically used to reduce sample complexity and to enrich for phosphorylated peptides prior to LC-MS analysis. There are different strategies available to purify phosphopeptides (Leitner, 2016; Low et al., 2020). Phosphopeptide enrichment using TiO₂-beads has proven to be an easy and robust method in our hands, which we employ using commercially available TiO₂-tips (High-Select TiO₂ phosphopeptide enrichment kit, Thermo Scientific). When necessary, this step can be replaced by any other phosphopeptide enrichment approach that yields phosphopeptides in similar purity and quantity. Subsequent stage-tip purification prior to MS-analysis is required as residual particulates or salts can negatively affect the C₁₈ columns and interfere with the analysis (Rappsilber et al., 2007).

33. Resuspend the dried peptides (95% v/v of previous elution) in 150 μL of Binding/Equilibration buffer.
 - a. Vortex the sample briefly and spin down in a tabletop centrifuge.
 - b. Ultrasonicate the sample in a water bath for 1 min at room temperature and spin down in a tabletop centrifuge.
 - c. To ensure binding, control for acidic pH of the resuspended sample using pH-paper (at or below pH 2).
34. Perform the phosphopeptide-enrichment as described in the manufacturer's protocol.
35. Dry the samples in a speedVac at room temperature.
36. Prepare stage tips with a 200 μL pipette tip and two disks of C₁₈ material for each sample (for details see Rappsilber et al., 2007).
37. Dissolve phosphopeptides in 0.1% aqueous FA.
 - a. Vortex the sample briefly and spin down in a tabletop centrifuge.
 - b. Ultrasonicate the sample in a water bath for 1 min and spin down in a tabletop centrifuge.
38. Condition the disks by adding 100 μL pure methanol. Centrifuge at 2,000 × g for 3 min.
39. Wash with 100 μL of 50% ACN/ 0.5% acetic acid. Centrifuge at 2,000 × g for 3 min.
40. Equilibrate with 100 μL of 0.5% acetic acid. Repeat this step once.
41. Load phosphopeptides onto the tip. Centrifuge at 1,500 × g for 5 min.
42. Wash and desalt with 100 μL of 0.5% acetic acid. Centrifuge at 2,000 × g for 3 min.
43. Elute the desalted phosphopeptides with 75 μL of 50% acetonitrile/ 0.5% acetic acid into a fresh 1.5 mL reaction tube. Centrifuge at 2,000 × g for 3 min. Repeat this step once.
44. Dry the sample in a speedVac at room temperature.

Note: To maximize your yield (troubleshooting 3), we recommend repeating step 41 and re-loading the phosphopeptides. For this purpose, use a fresh reaction tube before loading and re-add the solution that has passed the tip.

△ CRITICAL: Duration of the centrifugation steps during the phosphopeptide purification (steps 38–43) can vary. Generally, centrifuge until all solvents passed the membrane, while preventing it from drying out completely.

▮▮ Pause point: Purified and dried phosphopeptides can be stored at –20°C for at least 6 month. Do not freeze the peptides in the elution buffer after TiO₂-affinity enrichment. The basic pH can lead to a loss of the phosphate moiety from the modified peptides.

LC-MS/MS analysis

⌚ **Timing:** 1 day–1 week; depending on samples

The purified peptides are separated using reverse-phase (RP) chromatography, ionized via electrospray ionization (ESI) and analysed in the mass spectrometer. Peptide mass is determined in full

scans (MS1). The acquisition of fragment spectra (MS2) then allows unambiguous assignment of peptide identity and phospho-site location. This protocol is designed as a label-free, bottom-up approach, meaning that MS1 signal intensities of peptides are quantified and compared between experimental conditions, i.e., a treated and an untreated (control) sample.

Alternatives: The following steps are optimized for our LC-MS setup but any equivalent nano-HPLC system coupled to a high-resolution tandem mass spectrometer applicable for high-throughput proteomics can be used instead (e.g., Thermo Q Exactive, Exploris or Tribrid or Bruker TimsTOF Pro series).

45. Dissolve the dried peptides of each sample in 15 μL reconstitution buffer.
 - a. Vortex the sample briefly and spin down in a tabletop centrifuge.
 - b. Ultrasonicate the sample in a water bath for 1 min and spin down in a tabletop centrifuge.
46. Transfer the samples in autosampler vials, e.g., QuanRecovery vials (Waters).
47. Inject and load 3 μL of the reconstituted samples on C_{18} columns (e.g., trapping column: particle size = 3 μm , C_{18} , length = 20 mm; analytical column: particle size = 1.7 μm , C_{18} , length = 50 cm) using an Ultimate 3000 RSLC nano (using the LC-gradients described in the “materials and equipment” section).
48. Ionize the eluting peptides via a nanoESI source and detect them using a Thermo Scientific Fusion Lumos mass spectrometer (using the MS-parameters described in the “materials and equipment” section).

Raw data processing

⌚ Timing: 1 day–1 week; depending on samples

To extract quantitative information, MS raw-files are processed using dedicated software tools. In this protocol we describe the use of the open-source program MaxQuant (Cox and Mann, 2008), but any other proteomics software platform (e.g., Proteome Discoverer, PEAKS, MS Fragger, MetaMorpheus) can be used alternatively.

49. Process raw files using the open-source software MaxQuant. Data analyses of the phosphoproteome and the total proteome are performed in separate sessions. Differences in the analyses are stated in the text. If not indicated otherwise, the MaxQuant default parameters are used.
 - a. Import the raw-files of the phosphoproteome or total proteome (“Raw data” > “Load”).
 - b. Assign a distinct sample name for each file (“Raw data” > “Set experiment”) indicating condition and replicate.
 - c. Download a sequence database for *Rattus norvegicus* from UniProtKB (uniprot.org) and import it into MaxQuant (“Global parameters” > “Sequences”).
 - d. Set false-discovery rates (FDR) to 0.01 on protein, peptide and site level and a minimum of one unique peptide for protein identification (“Global parameters” > “Identification”).
 - e. Use a precursor mass tolerance of 4.5 ppm (“Group-specific parameters” > “Instrument”). For MS2 spectra acquired in the ion trap, use a fragment mass tolerance of 0.5 Da (“Global parameters” > “MS/MS - ITMS”). For MS2 spectra acquired in the orbitrap, use a fragment mass tolerance of 20 ppm (“Global parameters” > “MS/MS - FTMS”).
 - f. For total proteome analyses, quantify proteins in a label-free approach (LFQ) using unique peptides. To perform pair-wise ratio determination, use at least two common peptides in at least three consecutive full scans identification (“Global parameters” > “Protein quantification”; “Group-specific parameters” > “Instrument”).
 - g. For phosphoproteome analyses, assign variable phosphorylation on serine, threonine and tyrosine (“Group-specific parameters” > “Modification”).
50. Use the “phospho(STY)site” output-table to analyse the phosphoproteome on the level of phosphorylation events.

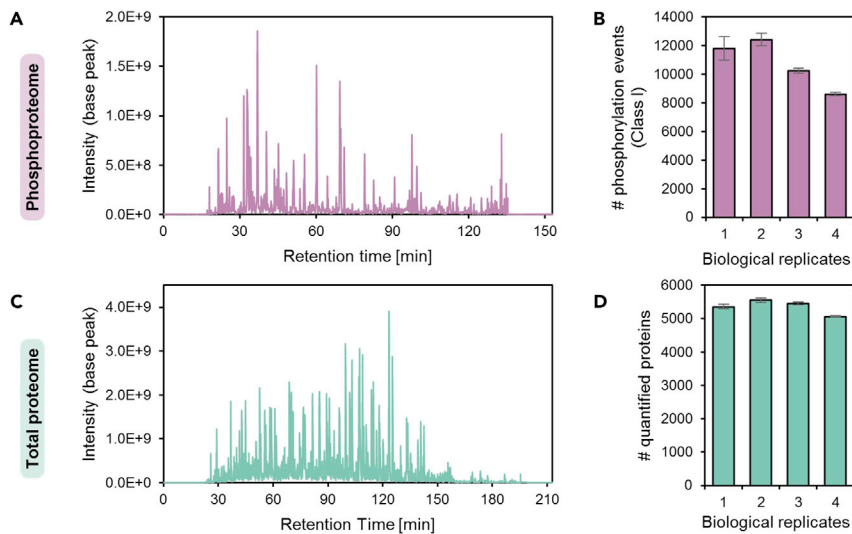


Figure 3. Results of exemplary phosphoproteomics or proteomics LC-MS runs

Base peak chromatograms of exemplary LC-MS runs of injected phosphopeptides (A) or unenriched peptides (C). For each analysis, 3 μ L of the reconstituted peptides were injected into the mass spectrometer.

To monitor reproducibility of the approach, we show the average number of identified phosphorylation events (B) or proteins (D) of three technical replicates in four independent biological preparations. Data were filtered for contaminants and decoy database hits. Phosphorylation events were further filtered for a location probability of the phosphorylated residue greater than 75% (so called Class I events; Olsen et al., 2006). Error bars represent the standard deviation of technical triplicate runs.

51. Use the “proteinGroups” output-table to analyse the total proteome on the level of proteins.

Note: The processing time of MaxQuant depends on the number and size of the raw-files, accessible computer power and speed of the hard drive or network (if files are not stored locally). The number of threads or processors used for computation can be adjusted manually. Each thread requires a size of at least 2 GB RAM.

EXPECTED OUTCOMES

In a typical bottom-up, label-free (phospho-)proteomics experiment, several thousand phosphorylation events and proteins can be identified and quantified. Depending on the number of conditions and runs, the overall number of phospho-event or protein identifications of one study can vary. With our LC-MS setup and samples prepared according to the presented protocol, we were able to quantify over 40,000 different phosphorylation events (Class I; total dataset) with around 11,000 events per individual LC-MS run (Figures 3A and 3B). Phosphoenrichment efficiency was achieved with a specificity greater than 90% (phosphorylated over total peptides). For the total proteome, we managed to characterize approximately 6,800 proteins overall with around 5,000 proteins per individual run (Figures 3C and 3D).

QUANTIFICATION AND STATISTICAL ANALYSIS

Comparative proteomic data can be analysed with a variety of different computational platforms, such as the open-source software Perseus (Tyanova et al., 2016) or any equivalent, i.e., the statistical computing environment R. In general, the first step of the downstream analyses is filtering of the data for contaminants, decoy database hits or - in case of the total proteome - for proteins only identified by a modified peptide. All entries matching these categories are marked in the corresponding output-tables and discarded. For the phosphoproteomics data, one further filter is for site localization probability greater than 75% (Class I events) to trace the modification back to a single amino acid residue. To assess phosphopeptide species of different multiplicity, the phosphorylation site

table of MaxQuant is then rearranged, i.e., by expansion of entries so that the number of phosphorylation sites per peptide is formatted as separate rows. Species within this table are referred to as phosphorylation events, since there can be more than one entry for one particular phosphorylation site, e.g., derived from a singly, doubly or triply phosphopeptide species.

For quantitative analyses, intensities of phosphorylation events and proteins are \log_2 -transformed and normalized according to the sample's median intensity to adjust for experimental variation such as unequal peptide load. Statistical comparisons are generally performed for treatment-control pairs of each tested time point. To mitigate the problem of missing values during statistical analyses, we filtered for valid values in all biological replicates quantified in at least one technical replicate. Differential regulation can be assessed using different statistical tests depending on the study design (e.g., unpaired, two-sided t-tests or ANOVA). For a pairwise comparison, we recommend implementing a linear mixed effect model using either the R-package lme4 (Bates et al., 2015) or MSqRob, an already established algorithm for label-free MS-experiments (Goeminne et al., 2018). In the mixed model, the treatment in question is set as the fixed effect and the biological replicates as a random effect (Desch et al., 2021; Dörrbaum et al., 2020). To obtain p-values, likelihood ratio tests of the model including the effect of interest against the model without it are performed. To correct for multiple testing, Benjamini-Hochberg correction is applied with an FDR cut-off < 0.01 (Benjamini and Hochberg, 1995).

The identified differential phosphorylation can be an independent regulatory mechanism but also an integration of both the changes in phosphorylation and protein expression (Wu et al., 2011). As both mechanisms have been quantified in our two-level analysis, we recommend investigating potential contribution of protein abundance to the regulated phosphorylation events. For this purpose, the overlap of the regulated proteome and regulated phosphorylation events is analysed by matching via protein identifiers. Further, the amplitude of regulation can be compared for any overlapping subsets, e.g., by plotting the fold changes of the intersection of regulated phosphorylation events versus the fold changes of the corresponding regulated protein. This way, changes in phosphopeptide abundance arising from differential phosphorylation rather than proteome abundance changes can be assigned.

LIMITATIONS

Before beginning with the (phospho-)proteomics protocol, we introduced primary cortical cultures as a model system to study neurons with fully-developed axons and dendrites in near-physiological conditions. One consideration working with this system is the contribution of other cell types, such as glia cells, to the total cell population ("mixed culture"). While we found that neurons and their proteins are dominant in culture, it's important to acknowledge the mixed population and consider including a cell-type specific analysis of (phospho-)regulated proteins in one's dataset (e.g., compare with Sharma et al., 2015). There are strategies to mitigate glia-contamination either by de-enrichment of this cell type using chemical treatments (Dörrbaum et al., 2018; Sharma et al., 2015) or techniques to separate subsets of proteins of a particular cell-types, e.g., via cell-type specific, metabolic labeling (Alvarez-Castelao et al., 2019). Strategies such as laser microdissection or fluorescence-activated cell sorting of labelled cells can be an elegant yet technically challenging option to implement considering the neuron's branched morphology which might result in loss of distal processes and synapses and the overall limited protein yield for proteomics sample preparation followed by phosphopeptide enrichment.

Regarding the step-by-step protocol, we present a label-free proteomics strategy with all its benefits and limitations. On one hand, this approach is very cost-efficient and easily implemented. In addition, the experimenter has flexibility in the design of the study (i.e., no introduction of SILAC amino acids in the cell culture medium) and no modification of the peptides potentially affecting their physicochemical properties is required (i.e., no modification of (phospho-)peptides using e.g.,

TMT-reagents). On the other hand, its reproducibility heavily depends on robust sample preparation and a well-maintained LC-MS system. Sample preparation, in particular the enrichment for low-abundant phosphorylated peptides, can introduce preparational variability. Hence, it is recommended to minimize any external variabilities within one study (i.e., different batches of C₁₈ cartridges or TiO₂-tips). We designed this protocol to mitigate these caveats and further provide additional recommendations in the “troubleshooting” section.

Another point of note is sample throughput: This protocol does not include isobaric tagging of (phospho)-peptides for sample multiplexing (Jones et al., 2020; Navarrete-Perea et al., 2018; Wu et al., 2021; Zecha et al., 2019). However, for large sample cohorts and/or if instrument time is limited, it can become necessary to use chemical tags (e.g., TMT-tags) to reduce overall measurement time. In this case, adaptation to the LC-MS workflow are required to ensure optimal data acquisition of the chemically modified peptides.

Phosphoproteomics studies using the workflow outlined here aim to survey phospho-events in great depth. This allows for a broad, untargeted and unbiased analyses of ten thousands of events. However, there is no guarantee that one will observe all phosphorylation events in an individual signaling cascade or will achieve information on “one favorite phosphorylation site”. If previous knowledge exists on specific phospho-signaling of interest or targets of differential phosphorylation have been reported previously, one should consider to design a targeted proteomics experiment, i.e., setting-up a parallel reaction monitoring (PRM) analysis (Peterson et al., 2012). If one aims to investigate PTM crosstalk or the dynamics of other PTMs (i.e., ubiquitination, acetylation, glycosylation), it can be necessary to perform additional enrichment experimental steps that are not covered in this protocol (i.e., via antibody-based pull downs; described elsewhere: Guan et al., 2010; Rivera et al., 2021; Udeshi et al., 2013).

TROUBLESHOOTING

Problem 1

Insufficient lysis of the cells.

Potential solution

An indicator of insufficient cell lysis is an opaque, inhomogeneous appearance of the lysate solution and a noticeable pellet after centrifugation (step 4). To improve lysis, the volume of the lysis buffer can be increased or additional mechanical disruption can be introduced. We recommend the use of ultrasonication, either in a water bath sonicator or via specialized devices such as the VialTweeter (Hielscher Ultrasonics). Another classical approach is the use of a plastic pestle to grind the cell pellet manually; however, this technique is generally prone to sample loss.

Problem 2

Impaired efficiency of tryptic protein digestion.

Potential solution

If undigested proteins are observed in LC-MS analyses (highly charged ions eluting in the high organic phase segment of the gradient) or if a high rate of missed-cleavage sites (20%–30% missed cleavages are within the expected range) is noted in the downstream data-analysis (i.e., in “evidence.txt” of MaxQuant output), this is an indicator of impaired protein digestion (Figure 4). The distribution of the cleavage sites and precursor charges should be comparable across all samples. To improve digest efficiency, the enzyme-to-protein ratio (step 18) can be adjusted. The protease LysC, that induces cleavage C-terminally to lysine residues, can be applied in parallel with trypsin. Further, one can consider carrying out the incubation of the overnight digest in an incubator at 37°C (step 20).

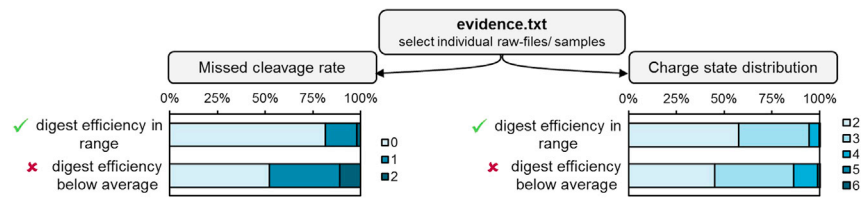


Figure 4. Evaluation of digest efficiency as part of the downstream data-analysis

The evidence-table is part of the output files generated by MaxQuant. It provides information on all identified peptides, i.e., the categories “missed cleavages” and “charge”. By plotting the number of missed, tryptic cleavage sites (left; 0–2 allowed) and the detected charge states of the precursor ions (right; 0–7 allowed), one can assess the digest efficiency for each sample analyzed in individual LC-MS/MS runs. A population of 70%–80% fully-tryptic peptides (with 0 missed cleavage sites) is well within the normal range. For trypsin-catalyzed digests, a precursor charge of 2 is often dominant in the charge state distribution ($z = 2$; i.e., one N-terminal charge plus the tryptic K/R-residue).

Problem 3

Poor yield of phosphopeptides following affinity-purification.

Potential solution

To allow for optimal enrichment of phosphorylated peptides, ensure that phosphatase inhibitors were used throughout cell harvest and lysis and that all buffers were fresh and at correct pH. To evaluate sample loss due to additional desalting of the phosphopeptides, one can consider skipping the additional phosphopeptide clean-up (steps 36–44). One needs to take into account that this can negatively affect the lifetime of your chromatography columns (in particular the trap column) and it is not recommended if you aim to analyse and compare larger sample cohorts.

Problem 4

Low number of phosphopeptide species exhibiting differential regulation.

Potential solution

The number of significantly regulated phosphopeptide species following data analysis depends on the applied treatment paradigm. A rather mild or specific treatment might affect only a few phosphoproteins, while a harsher perturbation can be less specific and results in a broader regulatory pattern with a more dynamic response (greater fold changes). Furthermore, the ability to detect differential phosphoregulation is linked to the experimental and statistical design of the experiment (Oberg and Vitek, 2009). Preparational steps such as the phosphopeptide enrichment are inherently prone to introduce variability (see “limitations” section). Hence subtle changes in phosphorylation levels between conditions can be hard to detect with sufficient certainty if the number of replicates is limited. To keep experimental variability to a minimum, we recommend to work with a minimum of four biological replicates and to perform the major preparational steps for all biological replicates together (ideally) using material from the same manufacturer’s batches. Regarding biological replication, we prepare replicate samples from independent neuronal preparations (separate litters).

RESOURCE AVAILABILITY

Lead contact

Further information and requests should be directed to and will be fulfilled by the lead contact, Julian D. Langer (julian.langer@brain.mpg.de).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The published protocol has been used to generate a phosphoproteomics dataset on homeostatic synaptic plasticity in primary cortical neurons. Data from this study were used to generate [Figure 3](#) (control samples of the 5 min time point). All data associated with this previous manuscript have been uploaded to the PRIDE repository and are available with the dataset identifier PXD021834 (RRID:SCR_003411; [Vizcaino et al., 2013](#)).

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AUTHOR CONTRIBUTIONS

K.D. designed the research protocol including associated data analyses and wrote the paper. J.D.L. and E.M.S. designed and supervised the project and co-wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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