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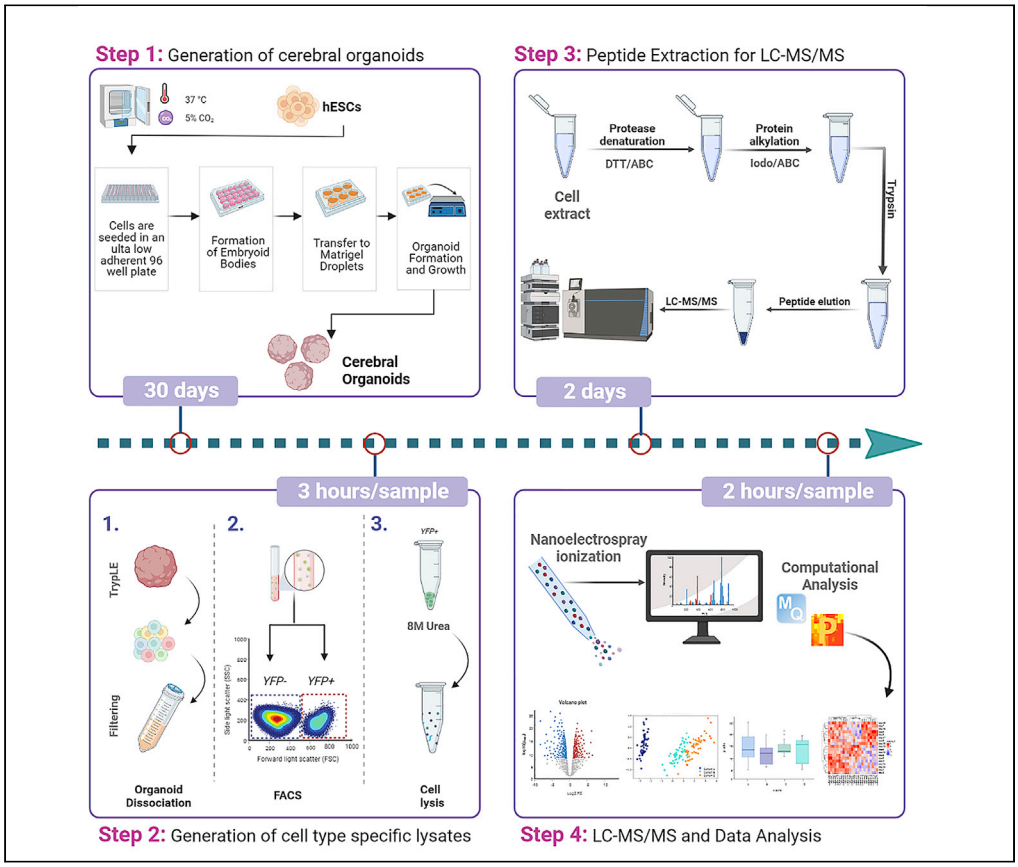
Generation of cell-type-specific proteomes of neurodevelopment from human cerebral organoids



Characterization of cerebral organoids has been challenging due to their heterogeneous nature. Here, we optimized a protocol to streamline the generation of FACS-purified cell populations from human cerebral organoids for proteomic analysis with liquid chromatography tandem mass spectrometry (LC-MS/MS). We describe the procedures for enzymatic dissociation of organoids into single-cell suspension, the generation of cell-type-specific lysates, peptide extraction, and proteomic analysis. This generalizable approach can be used to study temporal and cell-type-specific protein dynamics in developing cerebral organoids.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

A streamlined protocol covering human organoid cell sorting, lysis, and LC-MS/MS analysis

Enzymatic dissociation of organoids into single-cell suspension for downstream analysis

Generation of FACS-purified cell populations derived from cerebral organoids

Label-free quantification of cell-type-specific populations within organoid cultures

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Generation of cell-type-specific proteomes of neurodevelopment from human cerebral organoids

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SUMMARY

Characterization of cerebral organoids has been challenging due to their heterogeneous nature. Here, we optimized a protocol to streamline the generation of FACS-purified cell populations from human cerebral organoids for proteomic analysis with liquid chromatography tandem mass spectrometry (LC-MS/MS). We describe the procedures for enzymatic dissociation of organoids into single-cell suspension, the generation of cell-type-specific lysates, peptide extraction, and proteomic analysis. This generalizable approach can be used to study temporal and cell-type-specific protein dynamics in developing cerebral organoids.

For complete details on the use and execution of this protocol, please refer to Melliou et al. (2022).

BEFORE YOU BEGIN

Organoid culture and growth

Timing: 30 days

This protocol describes the methods involved in the proteomic analysis of cell type specific cell populations derived from cerebral organoids. While existing organoid culture protocols differ significantly from one to another, our protocol was developed using one of the most popular approaches in the field (Lancaster et al., 2013). Specifically, we utilized two genetically engineered human embryonic stem cell (hESC) lines where yellow citrine fluorescent protein (YFP) had been fused with the SRY-Box Transcription factor 2 *(SOX2)* and Doublecortin *(DCX)* genes respectively. We were particularly interested in these markers as *SOX2* is involved in the earliest stages of brain development, especially for neuroectodermal differentiation. In human tissue, this marker shows a high and restricted expression pattern in the neural stem and precursor cells, populating the ventricular zone of the developing neural tube (Mercurio et al., 2019). Conversely, *DCX* is later expressed in newly formed and differentiating neuroblasts, where it plays a role in their radial migration to form the neuronal layers found in cerebral cortex (Ayanlaja et al., 2017). Growing cerebral organoids derived from these tagged lines enabled the enrichment of precursor (SOX2+) and more differentiated (DCX+) cell compartments of organoids prior to profiling. Similarly, this protocol can be adjusted to accommodate other fluorescently tagged gene(s) of interest. For details of the generation cerebral

organoids, troubleshooting and tips please refer to (Lancaster et al., 2013; Lancaster and Knoblich, 2014).

Note: The techniques we describe here use organoids grown in an artificial scaffold made of Matrigel in 6-well tissue culture plates.

⚠ CRITICAL: While Matrigel is a liquid at 4°C, it solidifies at 20°C–25°C. Therefore, it is important to work on ice with pre-chilled reagents and pipette tips.

⚠ CRITICAL: The use of human tissue for research purposes is subject to ethical and legislative restrictions, appropriate approvals must be obtained before commencing work.

Institutional permissions

Any experiments on human tissue must be performed in accordance with relevant institutional and national guidelines and regulations. For our work, all experiments conform to the relevant regulatory standards and the appropriate stem cell research oversight committee (ROC) has approved all relevant experiments.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
DMEM/F12	Thermo Fisher Scientific	Cat#11320033
KnockOut Serum Replacement (KOSR)	Thermo Fisher Scientific	Cat#10828028
CTS GlutaMAX-I Supplement	Thermo Fisher Scientific	Cat#A1286001
Penicillin-Streptomycin (P/S)	Thermo Fisher Scientific	Cat#15140122
MEM Non-Essential Amino Acids Solution (100×)	Thermo Fisher Scientific	Cat#11140-050
2-Mercaptoethanol 50 mM	Thermo Fisher Scientific	Cat#31350-010
Recombinant Human FGF-basic	PeproTech	Cat#100-18B
Heparin sodium salt	Sigma-Aldrich	Cat#H3149-100KU
Y-27632 (Dihydrochloride)	STEMCELL Technologies	Cat#72304
N-2 Supplement (100×)	Thermo Fisher Scientific	Cat#17502048
B-27™ Supplement (50×), serum free	Thermo Fisher Scientific	Cat#17504044
B-27™ Supplement (50×), minus vitamin A	Thermo Fisher Scientific	Cat#12587010
Neurobasal Medium	Thermo Fisher Scientific	Cat#21103-049
Insulin solution human	Sigma-Aldrich	Cat#I9278-5ML
L-Ascorbic acid	Sigma-Aldrich	Cat#A4403-100MG
Fetal Bovine Serum, embryonic stem cell-qualified	Thermo Fisher Scientific	Cat#16-141-061
Matrigel Matrix hESC-Qualified Mouse 5 mL	Corning	Cat#354277
Matrigel Basement Membrane Matrix, LDEV-Free, 10 mL	Corning	Cat#354234
Chir99021, ≥98% (hplc)	Sigma-Aldrich	Cat#SML1046-25MG
Dimethyl sulfoxide (DMSO), Hybri-Max	Sigma-Aldrich	Cat#D2650-100ML
Gentle Cell Dissociation Reagent	STEMCELL Technologies	Cat#100-0485
mTeSR 1	STEMCELL Technologies	Cat#85850
Tryple Express Enzyme (1×), phenol red	Thermo Fisher Scientific	Cat#12-605-028
Bovine Serum Albumin Fraction V	Sigma-Aldrich	Cat#10735086001
D-PBS without calcium chloride and magnesium chloride	Sigma-Aldrich	Cat#D8537-500ML
DAPI Solution	Thermo Scientific	Cat#62248
Paraformaldehyde, powder, 95%	Sigma-Aldrich	Cat#158127-100G

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Urea, BioUltra, ≥99.5% (T)	Sigma-Aldrich	Cat#51456-2.5KG
Ammonium bicarbonate, BioUltra, ≥99.5% (T)	Sigma-Aldrich	Cat#09830-500G
Complete™ ULTRA Tablets, Mini, EASYpack Protease Inhibitor Cocktail	Sigma-Aldrich	Cat#5892970001
DL-Dithiothreitol,BioUltra	Sigma-Aldrich	Cat#43815-1G
Iodoacetamide,BioUltra	Sigma-Aldrich	Cat#I1149-5G
Trypsin/Lys-C Mix, Mass Spec Grade	Promega	Cat#V5073
Trifluoroacetic Acid Optima™ LC/MS Grade	Thermo Scientific	Cat#A116-50
Acetonitrile, Optima LC/MS Grade	Thermo Scientific	Cat#A955-4
Formic Acid	Thermo Scientific	Cat#A117-50
Pierce™ Water, LC-MS Grade	Thermo Scientific	Cat#51140
Critical commercial assays		
Pierce BCA Protein Assay Kit	Thermo Scientific	Cat#23225
Click-iT Plus TUNEL Assay	Thermo Scientific	Cat#C10619
Experimental models: Cell lines		
WA09	WiCell	RRID: CVCL_9773
AI03e-DCXYFP	WiCell	RRID: CVCL_UB70
AI06e-SOX2YFP	WiCell	RRID: CVCL_UB71
Software and algorithms		
MaxQuant Andromeda	Cox and Mann (2008)	https://maxquant.net/ RRID: SCR_014485 www.coxdocs.org
Perseus version 1.6.07	Tyanova et al. (2016)	https://maxquant.net/perseus/ RRID: SCR_015753
BD FACSChorus	BD Biosciences	NA
Xcalibur (v4.3.73.11)	Thermo Fisher Scientific	https://www.thermofisher.com/order/catalog/product/OPTON-30487
Leica Application Suite X software	https://www.leica-microsystems.com/products/microscope-software/p/leica-las-x-ls/	RRID: SCR_013673
Biorender	www.biorender.com	NA
Adobe Illustrator 2020	https://www.adobe.com/products/illustrator.html	RRID: SCR_010279
Other		
Ultra-Low Attachment, 96 Well Plate, Round Bottom	Corning	Cat#7007
Ultra-Low Attachment, 24 Well Plate, Flat Bottom	Corning	Cat#3473
Ultra-Low Attachment, 6 Well Plate, Flat Bottom	Corning	Cat#3471
BD FACSAria™ Fusion Flow Cytometer	BD Biosciences	RRID: SCR_018091
EASY-nLC 1000	Thermo Scientific	LC120
Q Exactive HF-X Hybrid Quadrupole-Orbitrap Mass Spectrometer	Thermo Scientific	IQLAAEGAAPFALGMBDK

MATERIALS AND EQUIPMENT

Preparation of media

Organoid Media

Medium	Day of differentiation	Components
Seeding Medium	Day 0 (Embryoid formation)	DMEM/F-12 + KOSR + ES-quality FBS + 1% (v/v) GlutaMAX + 1% (v/v) MEM-NEAA + 50 mM 2-ME + 1% P/S, 4 ng/mL bFGF, 5 mM ROCK inhibitor (Y-27632)

(*Continued on next page*)

Continued		
Medium	Day of differentiation	Components
Embryoid Body Media	Day 3 (Germ layer differentiation)	DMEM/F-12 + KOSR + ES-quality FBS + 1% (v/v) GlutaMAX + 1% (v/v) MEM-NEAA + 50 mM 2-ME + 1% P/S
Neural Induction Media	Day 5 (Neuroepithelia induction)	DMEM/F-12 + 1× N-2 + 1% (v/v) GlutaMAX + 1% (v/v) MEM-NEAA + 1 mg/mL Heparin + 1% P/S
Improved Differentiation Media-A	Day 13 (Expansion of neuroepithelium)	DMEM/F-12 + Neurobasal + 1× N-2 + 1× B-27 minus vitamin A + 9.5–11.5 mg/mL insulin + 50 mM 2-ME + 1% (v/v) GlutaMAX + 1% (v/v) MEM-NEAA + 1% P/S
Improved Differentiation Media+A+1.0 mg/mL $NaHCO_3$	Day 18 (Expansion of neuroepithelium)	DMEM/F-12 + Neurobasal + 1× N-2 + 1× B-27 plus vitamin A + 9.5–11.5 mg/mL insulin + 50 mM 2-ME + 1% (v/v) GlutaMAX + 1% (v/v) MEM-NEAA + 1.0 mg/mL NaHCO3, 40 mM L-ascorbic acid + 1% P/S
Improved Differentiation Media+A+1.5 mg/mL $NaHCO_3$	Day 30 (Cerebral tissue growth)	DMEM/F-12 + Neurobasal + 1× N-2 + 1× B-27 plus vitamin A + insulin + 50 mM 2-ME + GlutaMAX + MEM-NEAA + 1.5 mg/mL NaHCO3, 40 mM ascorbic acid + 1% P/S

Note: Store media at 4°C for a maximum of 1 month in sterile conditions.

Alternatives: Alternative reagents may be used but optimization might be required prior to cell type specific enrichment and proteomic analysis.

8 M Urea Buffer		
Reagent	Final concentration (mM)	Amount
Urea	8,000	4.80 g
Ammonium bicarbonate	50	0.04 g
LC-MS grade water	N/A	To 10 mL
Total	**8,000**	**10 mL**

Note: This solution is prepared fresh immediately before use.

FACS setup

Table 1.

Table 1. FACS instrument setup for BD FACSAria Fusion Cell Sorter

Parameter	Value
Drop Delay	24.65
Frequency	25.90
Sample Pressure	20 psi
Nozzle Size	100 μm
Area Scaling	FSC (0.70), Blue (0.70), Violet (1.01), Green (0.74), Red (0.83)
Channels	B515 (for YFP) V450 (for DAPI)

LC-MS/MS system setup

- Peptides are loaded onto a trap column, that is packed *in house* at 300 nL/min with an increasing concentration of 0.1% formic acid in acetonitrile, along a 90 min gradient and ending in a 15 cm long PicoTip Emitter (75 μm inner diameter, 8 μm tip, New Objective) with 3 μm Pursuit C18 (Agilent Technologies).
- A 90 min data-dependent acquisition (DDA) method is used. The mass spectrometer is set to perform full MS1 with a resolution of 70,000 in profile mode, over a mass range of 400–1,500 m/z. Following subsequent fragmentation, the top 28 parent ions of each spectrum are selected for MS/MS analysis, using the HCD cell and detection of fragment ions in the Orbitrap at a resolution of 17,500. Table 2.

Table 2. LC-MS/MS parameters	
Parameter	Value
MS1 Automatic Gain Control (AGC)	3e6
Maximum injection time (IT) for MS1 AGS	100 ms
MS2 AGS	1e5
Maximum IT for MS2 AGS	50 ms
Capillary temperature	275°C
Spray Voltage	2kV
Isolation Window	1.6 Da
Underfill ratio	2%
Intensity threshold	4e4
Normalized collision energy (NCE)	25
Charge exclusion	fragment only 2+,3+ and 4+ charge state ions
Apex trigger	Deactivated
Peptide match	Preferred
Dynamic exclusion	45 s

- Spectrum data are acquired with Xcalibur software version v4.3.73.11.

Alternatives: This protocol is optimized for our LC-MS/MS shotgun approach using a nano liquid chromatography setup that was coupled to Q Exactive HFX (Thermo Fisher Scientific) mass spectrometer. The ionization source in the system is nanoelectrospray (Thermo Fisher Scientific). Alternative systems with similar features should be suitable after optimization (Djuric et al., 2017).

⚠ CRITICAL: The instrument should be calibrated regularly, and its performance should be validated with *in house* quality controls prior to any sample analysis.

MaxQuant setup

Table 3.

Table 3. Max Quant parameters	
Parameter	Value
Group — Specific Parameters	
Digestion Mode	trypsin/P enzyme with up to 2 missed cleavages allowed
Variable Modifications	oxidation of methionine and N-terminal protein acetylation
Fixed Modification	carbamidomethylation of cysteine
LFQ minimum-ratio count	1
Global Parameters	
Identification	protein level false-discover rate (FDR) was set at 1%; match between runs

STEP-BY-STEP METHOD DETAILS

Organoid dissociation

Timing: 2 h

This section describes a step-by-step protocol for the enzymatic dissociation of cerebral organoids into a single cell suspension for downstream analysis. The exact timepoint to take organoids out of the culture will depend on the expression levels of the peptides of interest. In our work, we have used cerebral organoids that were 4–8 weeks old (Melliou et al., 2022). However, we have tested our protocol for organoid cultures up to 10 weeks old. Interestingly, we found that the DCX positive compartment show more dynamic temporal changes than the SOX2 positive

populations across the collected 2-week intervals. This observation will vary according to the cell biology of the specific population/marker chosen.

1. Transfer the cerebral organoids from the 6-well plate used for culturing to a 5 mL falcon tube, separated by condition.

   ⚠ CRITICAL: Only healthy organoids should be used for organoid dissociation and further analysis. Ensure that hESCs show healthy stem cell morphology and are 70%–80% confluent before seeding. In addition, observe cultures regularly under light microscope and monitor the growth and development of cerebral organoids after every critical step (embryoid body formation, neuroepithelium induction, organoid formation, etc.) as it is previously discussed in (Lancaster and Knoblich, 2014), (Figure 1). Although, cerebral organoids can be variable within and between the batches, proper formation is necessary in order to better investigate their molecular proteomic patterns in a reproducible manner (Problem 1).

   Note: This step should be performed using a cut 1,000 μL tip, to avoid disruption of the organoid.

   Note: You can use 5–7 organoids per falcon tube, depending on their size and age. In our experience, organoids growing for more than 10 weeks are harder to dissociate with the conditions described in this section (Problem 2).

2. Remove excess medium from each tube and wash with cold PBS (1×) three times.

   Note: Using cold PBS serves as an effective way not only to remove traces of culture medium, but also to mechanically remove the extracellular matrix (Matrigel) that surrounds cerebral organoids and reduces peptide degradation. Remaining matrix may interfere with peptide

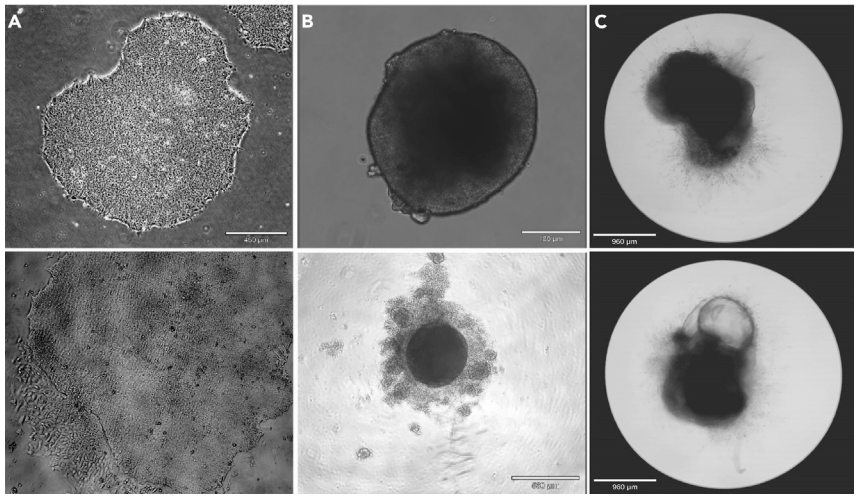

Figure 1. Proper formation and differentiation of cerebral organoids

Examples of appropriate and poor cultures at different stages.

(A) Brightfield image of a healthy (top) and unhealthy (bottom) hESC colony prior to seeding. The scale bar represents 460 μm.

(B) Brightfield image of successful (top) and failed (bottom) formation of embryoid body 5 days after plating into single cell suspension. The scale bar represents 180 μm and 360 μm respectively.

(C) Brightfield image demonstrating an example of an acceptable organoid (top) for further analysis, and a failed organoid, that lacked to produce neuroepithelium and showed large fluid cysts structures (bottom). The scale bar represents 960 μm.

extraction and LC-MS/MS. Thus, cerebral organoids are advised to be washed with ice-cold PBS until no visible matrix remains (normally 2–3 times).

Alternatives: Other experimental approaches to eliminate interference of Matrigel contaminants efficiently can be used for proteomic sample preparation of organoids (Wang et al., 2022).

3. Remove PBS and add 1 mL TrypLE in each tube. Place all the tubes in a 360° rotating shaker for 30 min and incubate at 37°C with 5% CO_2.
   a. Using a 1,000 μL tip, pipette up and down vigorously to break the cerebral organoid in cell clumps and individual cells.
   b. Incubate for another 15 min.

Note: The incubation time with TrypLE varies and depends on the age and complexity of the organoid cultures (Problem 2). Alternative enzymes or commercially available dissociation kits with similar features should be suitable after optimization.

4. Add 2 mL of FACS buffer, containing PBS/FBS 98/2 (v/v) to stop the enzymatic reaction and pipette up and down to break down the last clumps with a 1,000 μL pipette tip.
5. Spin down the tubes (5 min in 200 × *g* at room temperature; 20°C–23°C). Resuspend the cell pellet in 1 mL of FACS buffer.
6. To remove remaining cell aggregates and non-dissociated tissue, pass the cell suspension through a 37 μm strainer.
   a. Moisture the strainer with 200 μL of FACS buffer before loading the cell suspension.
   b. Aspirate the resuspended cells in FACS buffer and dispense it over the top of the strainer and into the flow tube.

Note: It is important to add the solution in parts and push down on the mesh strainer slowly so you won't damage the strainer. It is also advised to tap and slowly pipette if needed before adding more of the solution.

7. Perform a viable cell count using trypan blue.

Note: To proceed with downstream analysis, it's advised to have more than 80% viability in all cell samples (Problem 3).

Cell sorting and lysis

Timing: 1 h/sample

These next steps describe how to set the compensation parameters and appropriate gates for multicolor FACS to isolate the cerebral organoid–derived cells by cell type (neurons and neural progenitors) based on their fluorescent reporters. In addition, we describe the subsequent lysis with 8 M urea for the downstream proteomic analysis.

8. Use unstained organoids as a negative control to determine optimal settings for gating and discerning positive from negative cells.

Note: Unstained control samples should be processed exactly as the stained ones, using the protocol described in the previous section.

9. Add a viability dye (such as DAPI, 1:100) in all samples, including the controls, to separate live from dead cells during cell sorting.

Note: The use of a viability marker is important since it could reduce noise and improves separation of cell populations.

10. Follow manufacturer instruction regarding use of cell sorters and sort the enriched (i.e., YFP+) and depleted (i.e., YFP-) populations. Certain parameters are also mentioned in Table 1.

Alternatives: Alternative tagged lines or fluorophores can be used to identify more than one cell population at a time. However, it is strongly recommended to optimize all the parameters and use all the necessary controls.

11. Collect cells in 15 mL falcon tubes with sorting efficiency of 90% or higher (Figure 2).

Note: The number of organoids required for fluorescence-activated cell sorting (FACS) must be determined based on the cell population(s) of interest; we recommend collecting >100,000 cells per sample for the downstream proteomic analysis (Problem 4).

12. Wash samples.
    a. Centrifuge at 1,000 × *g* for 3 min at 4°C.
    b. Remove supernatant without disturbing the pellet and wash with cold PBS.
    c. Centrifuge at 1,000 × *g* for 3 min at 4°C (optional to spin supernatant as well).
    d. Transfer samples to Eppendorf tubes.
    e. Centrifuge at 1,000 × *g* for 10 min at 4°C.
13. Cell lysis.
    a. Remove supernatant.
    b. Add around 200 µL of UREA lysis buffer.
    c. Incubate for 30 min in ice.
    d. Snap freeze in liquid nitrogen.

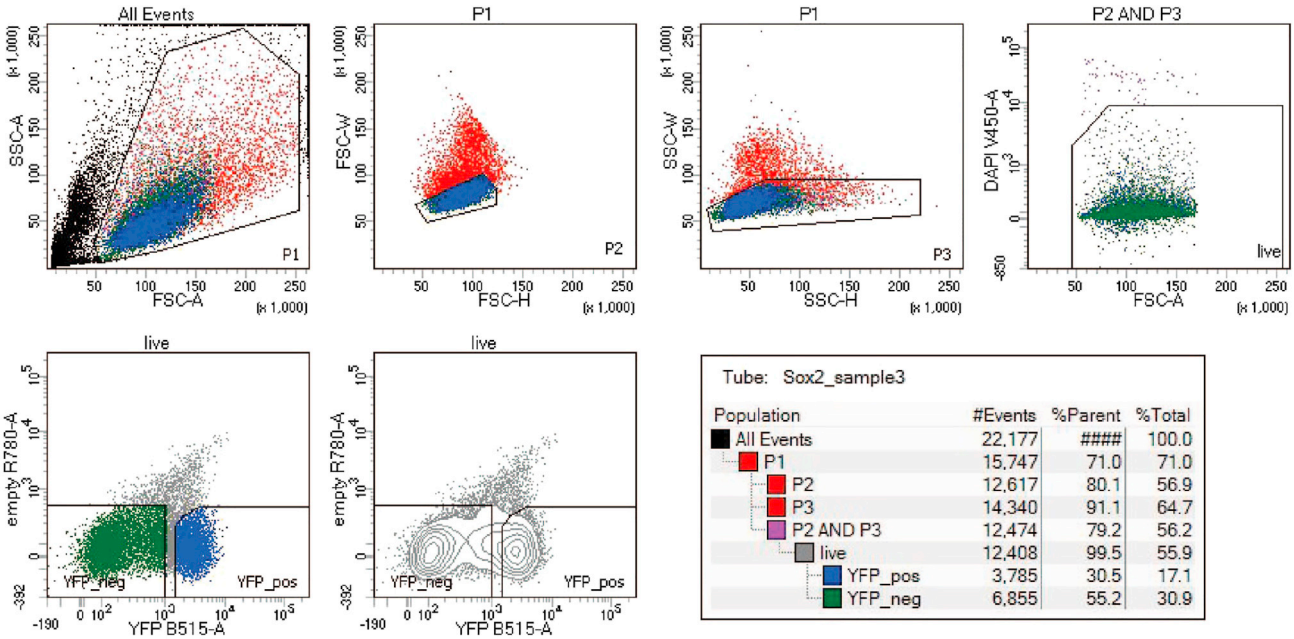

Figure 2. FACS analysis of single cells after dissociation using trypLE

Representative sort layout from 500k events, showing data from cerebral organoids expressing YFP. Single cells were selected based on their side scatter (SSC) and forward scatter (FSC) (top row) and live single cells were further isolated from dead cells and debris based on DAPI fluorescence as indicated. Cells were further distinguished from non-fluorescent negative (YFP-) cells (bottom row).

Note: Upon cell lysis, urea is the preferred cell lysis reagent because of its low cost (compared to RapiGest) and its high compatibility for mass spectrometry workflows. Indeed, it has been previously shown that users are able to achieve higher protein yields using Urea compared to RIPA for cell lysis (Ngoka, 2008).

Pause point: Samples can be frozen at −80°C until ready to continue with peptide extraction.

Peptide extraction

Timing: 2 days

This method describes peptide extraction of lysed and sorted cells derived from cerebral organoids.

14. Defrost the samples on ice.
15. Sonicate the samples, using a water bath sonicator (Bioruptor Pico) at 4°C to allow complete cell disruption and lysis.

Note: For this step we have followed a protocol with ultra-high (ultrasonic wave frequency 20–60 kHz), 30 s on, 30 s off for 30 min.

16. Centrifuge at 12,000 × *g* for 15 min at 4°C and transfer the supernatant to a clean Eppendorf tube.

Note: It is advised to keep the samples in ice at all times before proceeding with protein purification.

17. Quantitate the total protein by sample by running a BCA assay as per manufacturer instructions.

Note: It is required to have a minimum of 12 μg of protein concentration per sample for further analysis.

18. Top up the sample volumes to 100 μL with 50 mM ammonium bicarbonate.
19. Add 10 μL of 60 mM DTT in 50 mM ammonium bicarbonate for protein denaturation, to a final concentration of 6 mM.

Note: This solution is prepared fresh immediately before use. For its preparation personal protective equipment and the use of fume hood is required.

20. Incubate the samples on a thermoblock at 1,200 × *g* for 45 min at 45°C.
21. Add 11 μL of 100 mM iodoacetamide in 50 mM of ammonium bicarbonate for protein alkylation, to a final concentration of 18 mM.

⚠ CRITICAL: Iodoacetamide is unstable and light sensitive. Thus, this solution is prepared fresh immediately before use, and it is advised to perform alkylation in the dark.

22. Incubate the samples on a thermoblock at 1,200 × *g* for 45 min at room temperature in the dark.
23. Trypsinize all samples by adding Trypsin/Lys-C Mix, Mass Spec Grade (1:25 protein ratio).
24. Incubate the samples overnight (around 5–8 h in total) on a thermoblock at 1,200 × *g* at room temperature.
25. Dilute the samples with 13.8 μL of 10% trifluoroacetic acid (TFA) in 50 mM ammonium bicarbonate, for a final concentration of 1% (v/v).

⚠ CRITICAL: TFA is toxic and volatile. Therefore, use the fume hood when preparing the solution by adding the TFA first and then the ammonium bicarbonate.

26. Incubate the samples on a thermoblock at 1,200 × *g* for 20 min at room temperature.
27. Centrifuge at 13,000 × *g* for 10 min and transfer the supernatant to a clean Eppendorf tube.
28. By using Pierce C18 zip tip pipette tips, as per manufacturer instructions, the salts, lipids, DNA, and other contaminants are removed.
29. Elute the bound peptides with 3 μL of ACN/H_2O 65/35 (v/v) + 0.1% TFA.
30. Dilute the samples in 42 μL with H_2O + 0.1% TFA.

Proteomic analysis by LC-MS/MS

Timing: 90 min per sample

This step describes sample data acquisition of trypsinized samples by LC-MS/MS analysis. The samples are analyzed using a Thermo Fisher nano liquid chromatography setup that was coupled to Q Exactive HFX (Thermo Fisher Scientific) mass spectrometer using a nanoelectrospray ionization source. The analytical parameters are stated previously in Table 2.

31. Prior to injection onto the liquid chromatography system, centrifuge samples at 3,500 × *g* for 10 min.
32. Inject samples onto LC-MS/MS system for analysis.

Note: It is recommended to load a volume of 40 μL per sample, in order to have two technical replicates (18 μL per injection).

EXPECTED OUTCOMES

Our workflow allows the temporal study of protein-level differences within cell subpopulations derived from cerebral organoids. While transcriptomic efforts have shown that molecular signatures are changing in organoids in different developmental time periods, especially by activating cellular stress pathways (Bhaduri et al., 2020), similar proteomic efforts have been scarce. By generating organoids from genetically engineered hESC lines with endogenous fluorescently label gene tags, we could achieve enrichment prior to profiling. In a typical experiment starting from 5–7 organoids (total of 500,000–2,000,000 cells in total) per sample, with an overall sorting efficiency of 90%, we can expect to obtain 350,000–1,000,000 cells for the timepoints, and cell populations noted. In general, the expression of the marker in the population of interest at a certain timepoint of the organoid growth, is highly correlated with the yield of enriched cells. Thus, the versatility of our protocol allows the investigation of proteomic patterns of different markers present in organoids in multiple timepoints of their organoid growth. While our work has mostly focused on neuronal progenitors (SOX2+ cells), that arise very early in brain neurodevelopment, and immature and migrating neurons (DCS+ cells), we expect that other cell types from dissociated organoids can also be analyzed to investigate relevant molecular mechanisms of neurodevelopment and disease. In addition, utilizing technologies, such as FACS, allows the high purity (95%–100%) of the cell populations of interest, especially when we are interested in isolating cells based on internal staining or intracellular protein expression, such as a genetically modified fluorescent protein markers.

LC-MS/MS-based proteomics coupled with the brain organoid system provide new avenues of investigation in identifying neural lineage commitment and probe dynamic proteome changes across normal cellular differentiation states during human brain development and disease (Melliou et al., 2021). Indeed, one of the SOX2-enriched proteins (RuvB like AAA ATPase 2) that we identified and investigated in our original study, didn't appear to be enriched in this cell compartment at the mRNA level. For cerebral organoids that have been cultured up to 8 weeks of their organoid growth

you can expect identification of 2,000–7,000 proteins in total and quantification of around 2,000 proteins per sample when analyzed with shotgun LC-MS/MS proteomics.

The outlined shotgun approach also provided relevant protein abundances that are compared across samples. It is of note that these values are not quantitative reading, but rather relative amounts to the other samples being analyzed. The p value and fold change analysis depend on the statistics and bioinformatics utilized by the investigator and their biological question. In our workflow, you can expect the most abundant proteins to be the ones that can easily take up a charge (i.e., hydrophilic proteins), whereas the ones with the hydrophobic nature may be under-represented. Furthermore, when using the SOX2-YFP and DCX-YFP hESC lines, you can expect to identify proteins that are associated with gene ontology (GO) terms related to DNA replication and cell-cycle pathways and are highly enriched in the SOX+ population. Conversely the DCX+ populations, isolated from the DCX-YFP lines show proteins involved in neural differentiation-related pathways including axon development and neuron projection guidance. You can see examples of expected results in (Melliou et al., 2022) and Figure 3.

There is a high demand for an *in vitro* model to study the protein dynamics in different cell types involved in human brain development. Previous efforts have been focused on studying the developmental trajectory of human cerebral organoids at the bulk level. Our protocol advances these efforts by using a generalizable approach to investigate dynamic spatiotemporal changes in the post-transcriptional molecular landscape of developing cerebral organoids The long-term 3D organoid

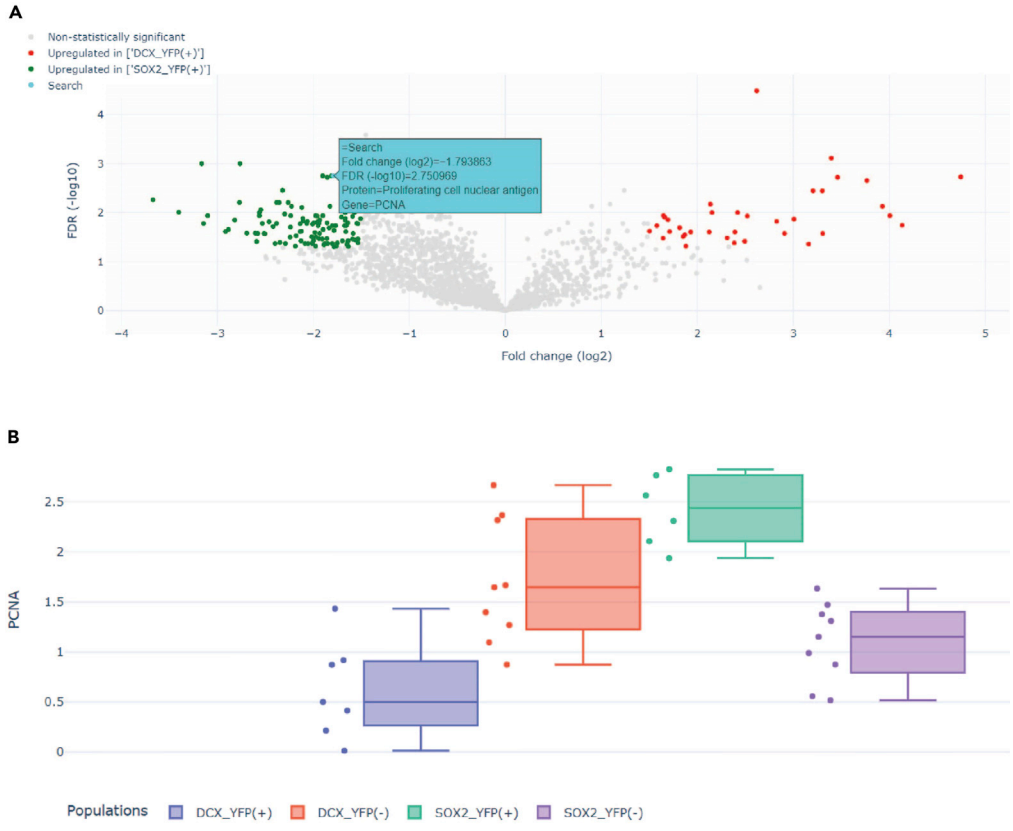

Figure 3. An example for visualizing the proteomic analysis of raw LC-MS/MS data files

(A) Volcano plot of differentially abundant proteins between SOX2+ (right) and DCX+ (left) organoid cell populations. Bonferroni-corrected p-value cutoff=0.1, Log2 Fold-change cutoff=1.333, n=13.

(B) Cell type specific differential expression of neural progenitor marker, PCNA, highlights enrichment within the SOX2+ (and DCX-) populations.

cultures and the molecular techniques utilized in this protocol may open new avenues to understand proteomic differences between cell populations, present in the organoid system that can better recapitulate the *in vivo* complexity of the human brain. In addition, its potential to highlight several proteins with timepoint specific expression patterns, can serve as a tool to understand specific molecular mechanisms involved in neurodevelopment and disease.

QUANTIFICATION AND STATISTICAL ANALYSIS

All mass spectrometry RAW files are uploaded into MaxQuant Andromeda search engine (www.coxdocs.org (Cox and Mann, 2008)) to quantify the mass spectrometry data against a Human SwissProt protein database (July, 2019 version). The analytical parameters are stated previously in Table 3.

LIMITATIONS

The organoid system

Due to the organoid system's current lack of the full diversity of neural cell types and vascular networks, not all cell types present in the human brain can be identified and enriched using this system and approach. It is advised to focus on cell-type-specific markers that are well known to be modeled within the organoid system of choice, in order to overcome this limitation. In addition, the inherent spatial and cellular heterogeneity of the system may also pose specific challenges, requiring the need of multiple replicates to successfully model subtle aspects of the brain biology of interest. While the approach of sorting and enriching organoid subpopulations using this method helps partly overcome this limitation, it's important to recognize that the negative population for the specific marker of interest will remain heterogeneous and much harder to interpret compared to the positive population.

This approach was designed to monitor the spatiotemporal molecular patterns of two distinct cell populations (i.e., neural progenitors and neurons) within cerebral organoids. However, the dynamic diversity of molecular signatures during the early stages of development of the human brain poses another limitation regarding the selection of the targeted markers. Therefore, markers which appear to have a highly dynamic expression pattern or may be differentially expressed in more than one cell populations may not be suitable for our workflow and a more targeted analysis is required with the use of multiple fluorophores to efficiently isolate more homogeneous cell populations within the organoids. Similarly, while longer culture times are likely to be successful, if the chosen marker loses expression with time, the yield of cell enrichment may be compromised.

Organoid cell dissociation

Extended incubation with TrypLE can cause increased cell death and low yield of viable single cells. At the same time, a longer incubation may be required for organoids that are older than 10 weeks. Other parameters such as the dissociation reagent, temperature of dissociation, and time of incubation may need to be optimized before processing with older organoids.

Proteomic analysis

While our proteomic analysis allowed the identification and quantification of a remarkable number of proteins, the technical reproducibility using a DDA method remains a challenge. Peptide ionization from electrospray ionization is preferential for polar side groups. Therefore, peptides containing hydrophilic side chains, such as arginine are more prevalent than hydrophobic side chains, such as alanine. Biologically this would result in an underrepresentation of membrane-associated peptides with repeating hydrophobic amino acid chains.

In addition, the most abundant precursor ions are selected for fragmentation resulting in a stochastic nature to data acquisition. Similarly, low-abundant proteins are underrepresented. This bias is particularly interesting with complex peptide mixtures such as organoid cultures, especially when they are undergoing cell dissociation and sorting before the molecular analysis. These methods

can create cell impairment and elevate stress related proteins, which may mask the proteome of certain populations.

Our workflow can be expanded in any genomic or proteomic workflow, including these with deeper fractionation-based proteomic approaches, and those that also capture relevant post-translational modifications. However, with these more advanced approaches more starting material and tandem mass tagging may be required to achieve deeper coverage with consequent increase in the budget and instrumental complexity.

TROUBLESHOOTING

Problem 1

Poor formation of organoid cultures.

Potential solution

Ensure that the hESCs look healthy and are 70%–80% confluent before seeding. Unhealthy cells can result improper formation of the organoids and increased debris in every step of their growth. Cultures should be kept in low attachment 6-well plates with no more than 8 organoids per well. To ensure proper circulation of media and oxygen, use orbital shakers for long term culture. See Figure 1 for an example of successful and unsuccessful organoid cultures.

Problem 2

Low yield or low viability of organoid dissociated cells. Especially older organoids (more than 10 weeks old) tend to be harder to dissociate, resulting in lower yield of cell numbers (step 3).

Potential solution

Our protocol is optimized to dissociate cells derived from cerebral organoids that are no older than 10 weeks old. To avoid low viability, refrain from pipetting too harshly, or with pipette tips smaller than 1,000 μL and optimize the incubation time of TrypLE. Longer incubation can result in low viability of the cells. It is strongly recommended to always filter samples before proceeding to FACS to remove cellular debris and other potential contaminants that can affect the sorting analysis. For organoids past 10 weeks, other dissociating reagents (enzymatic or mechanical) may be appropriate for high cell yield.

Problem 3

Low sorting efficiency (steps 6 and 7).

Potential solution

Low sorting efficiency might be a result of cells that are too concentrated and/or flow settings that could be too high for efficient sorting. To overcome this problem, you can reduce flow speed and/or dilute cell sample with more FACS buffer.

Problem 4

Low yield of enriched cell population for mass spectrometry (step 11).

Potential solution

Given that is challenging to harvest enriched cell populations that are sufficient for mass spectrometry applications, we suggest that you prepare replicates with 5–7 organoids (depending on their size) per sample. It's also important to identify early on the temporal expression of the marker during the organoid development so you can avoid timepoints that the intrinsic expression is inherently low. Pilot studies to establish the relative frequency of the cell population of interest is also helpful in planning accordingly to ensure sufficient cell counts will be recovered for proteomic analysis.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Phedias Diamandis (p.diamandis@mail.utoronto.ca).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The published article includes all datasets generated or analyzed during this study.

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AUTHOR CONTRIBUTIONS

S.M. and P.D. conceived the described approach. Organoid experiments and mass spectrometry of organoid sorted populations were carried by S.M. Interpretation of the data and writing of the manuscript was done by S.M. and P.D.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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