Quantitative Neuronal Cell-Type Proteomics Workflow for Neurodegenerative Disease Studies

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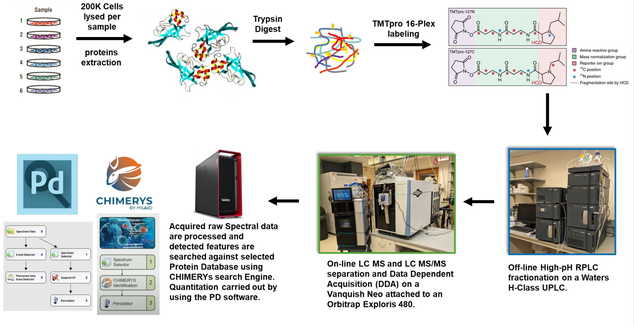
Introduction

Recent front-end automated samples preparation for SCP has paved the way for minimization of quantitative proteomics variability. Additionally, many current state-of-the-art MS in the market are capable of global proteomics detection of very low protein sample input (e.g., <50 picogram total digested protein on column), and are within the estimated range of 100-300 picogram total protein content in a single cell. Even with these SCP biotechnology advances; a major challenge still exists to apply these analyses workflows studying individual neuron. Specifically, difficulty in isolating biologically viable single neuron prior to proteomics sample preparation. We investigate TMT-labeled proteomics strategy to study neuronal cell-type proteomics (nCTP)™ of ALS patient's iPSC differentiated neurons.

Workflow



Cell Culture

Material

- The study included two cell lines: a control line (-"3920") and an ALS patient line (-"4307")
- 3 protocol were used in parallel and in separate plates

Motor neurons

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Efficient generation of lower induced motor neurons by coupling Ngn2 expression with developmental cues

Francesco Limone [1], Irune Guerra San Juan [2], Jana M Mitchell [3], Janell L M Smith [4], Kavya Raghunathan [1], Daniel Meyer [1], Sulagna Dia Ghosh [1], Alexander Couto [4], Joseph R Klim [3], Brian J Joseph [1], John Gold [4], Curtis J Mello [1], James Nemesh [1], Brittany M Smith [4], Matthijs Verhage [6], Steven A McCarroll [1], Olli Pietiläinen [1], Ralda Nehme [1], Kevin Eggan [10]

Cortical neurons

Neuroresource

Rapid Single-Step Induction of Functional Neurons from Human Pluripotent Stem Cells

Yingzhu Zhang [1], Changhui Pak [1,4], Yan Han [1,4], Henrik Ahlenius [1,4], Zhenjie Zhang [1], Soham Chanda [1,4], Samuela Marro [1,4], Christopher Patzke [1], Claudio Acuna [1], Jason Cory [1], Wei Xu [1,2], Nan Yang [1,4], Tomas Danko [1,4], Lu Chen [1], Marius Wernig [1,4], Thomas C. Südhof [1,2,3], X. EE

CRISPR Interference-Based Platform for Multimodal Genetic Screens in Human iPSC-Derived Neurons

Ruilin Tian [1], Mariam A Gachechiladze [2], Connor H Ludwig [3], Matthew T Laurie [4], Jason Y Hong [3], Diane Nathaniel [3], Anika V Prabhu [3], Michael S Fernandopulle [2], Rajan Patel [4], Mehnoosh Abshari [4], Michael E Ward [3], Martin Kampmann [2]

Microglia

iPSC-Derived Human Microglia-like Cells to Study Neurological Diseases

Edsel M Abud [1], Ricardo N Ramirez [2], Eric S Martinez [1], Luke M Healy [3], Cecilia H H Nguyen [1], Sean A Newman [4], Andriy V Yeromin [5], Vanessa M Scarfone [4], Samuel E Marsh [6], Cristhian Fimbres [1], Chad A Caraway [1], Gianna M Fote [1], Abdullah M Madany [6], Anshu Agrawal [3], Rakez Kayed [10], Karen H Gylys [11], Michael D Cahalan [5], Brian J Cummings [12], Jack P Antel [13], Ali Mortazavi [2], Monica J Carson [4], Wayne W Poon [14], Mathew Blurton-Jones [14]

Method

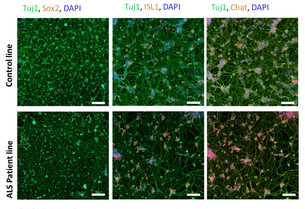
Cell Lysis: The cells were washed thrice with 1X PBS (Gibco, no calcium, no magnesium) and finally resuspended in 1X PBS. Cell concentrations were measured using an automated cell counter (BioRad). For each cell line, 200,000 cells were collected in LoBind 500mL tubes (Eppendorf) in duplicates and centrifuged at 10,000rpm. The supernatants were discarded, and cell pellets were stored at -80°C until processed further. Proteins were extracted using a lysis buffer composed 1X RIPA buffer (Cell Signaling Technology) containing phosphatase and protease inhibitors and SDS was added to a final concentration of 2%. To each cell pellet, 50mL lysis was added, and the cells were gently resuspended and heated at 95°C for 10 min. The cell lysates were sonicated in a water bath sonicator for 15 min. Lysed cells were centrifuged and cellular debris were removed.

Protein Extraction and Digestion: Proteins were reduced using 10mM DTT (Sigma Aldrich) at 60°C for 15 min and alkylated using 20mM IAA for 20 min in dark. Proteins were precipitated overnight at -20ºC using 5 volumes of chilled 100% acetone. Protein pellets were retrieved through centrifugation at 14,000 g for 15min at 4ºC. Fresh 80% acetone was added to the pellets and repeated for a total of 3 washes. The expected protein yields were calculated based on the number of cells collected in each tube and the enzyme : substrate ratios were adjusted. Enzymatic digestion was initiated by addition of Lys-C (Wako) and incubation at 37ºC for 4h. To the mixture, trypsin (Promega) was added and incubated overnight at 37ºC. The digestion was stopped by addition of equal volume of 1% TFA prepared in 99% isopropanol. The samples were centrifuged at 14,000 g for 15 min at 4ºC and supernatants were transferred to fresh microcentrifuge tubes for peptide clean-up.

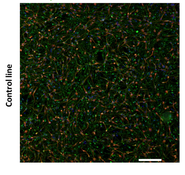
Peptide Clean-Up: Peptides were desalted using SDB-RPS StageTips (Empore). The dried peptides were reconstituted in 0.1% formic acid and equal volume of 1% TFA prepared in 99% isopropanol was added. The samples were vortexed and centrifuged at 14,000 g for 15 min at 4ºC. Supernatants were transferred to fresh microcentrifuge tubes and peptides were desalted using SDB-RPS StageTips (Empore). Pre-packed StageTips were washed using 100µL of 100% acetonitrile followed by 100µL of activation buffer (30% Me-OH+1%TFA) and then 150µL of 0.2% TFA. Samples were loaded twice onto the tips and washed using 150µL of 1% TFA in EtOAc followed by 100µL of 1% TFA in isopropanol. Peptides were eluted using 60µL of 1.25% NH_4OH in 80% acetonitrile and dried in speed vac.

TMTpro 16-Plex labelling: Dried peptides were reconstituted in 100mM TEABC and quantified using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific). The peptides (10mg) were labelled using a TMTpro 16plex Isobaric Label Reagent Set (ThermoFisher Scientific). The labels were reconstituted as per manufacturers' instructions and 20µL of each label was added to the tryptic peptides. Samples were mixed by vortex and incubated at room temperature for 1h with intermittent vortex. The labelling reactions were stopped by addition of 5µL of 5% hydroxylamine (ThermoFisher Scientific) and incubated for 15min at room temperature. 2mL of labelled peptides from each sample were pooled into a 0.5mL protein LoBind tubes (Eppendorf) and dried in a speed vac for label check efficiency. Remaining labelled peptides were pooled, dried, and stored at -80C until ready for offline fractionation. Dried pooled samples were reconstituted in water and cleaned up using a macro C18 spin column. A 2µL aliquot was saved for future LFQ tests. Pooled TMTpro 16-plex sample was then offline fractionated utilizing a high pH reverse phase C18 2.1mm column hooked up on a Waters H-Class UPLC. 40 x 1mL fractions were collected and staggered pooled into 10 pooled fractions and dried in a speed vac.

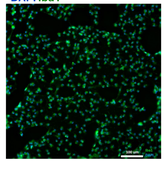
Results



iPSCs are maintained in mTeSR1 on Matrigel and seeded at high density in Y27632-supplemented media (Day 0). From Day 1, NGN2 induction begins with doxycycline and dual SMAD inhibition (SB431542, LDN193189), followed by caudalization with retinoic acid (RA) and ventralization using Smoothened agonist (SAG). Puromycin selection starts on Day 2. On Day 7, cells are dissociated and replated onto PDL/laminin-coated plates in neuronally supportive media (Neurobasal + B27, N2, neurotrophic factors). Media is refreshed every other day to support motor neuron maturation.

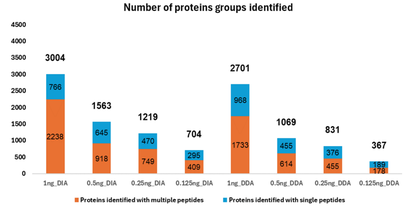


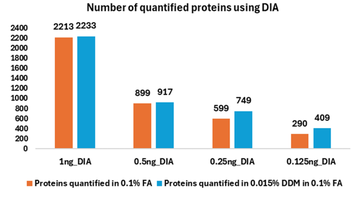
This is based on Zhang *et al*, 2013 and subsequently Tan *et al*, 2019 protocols. Dox-inducible NGN2 iPSCs are first cultured in mTeSR1 on Matrigel until ~80% confluent, then dissociated and replated in pre-differentiation medium containing doxycycline to induce NGN2 expression. After 3 days, cells are dissociated again and seeded onto PDL/laminin-coated plates in classic neuronal medium. Day 0 is marked at this point. Doxycycline is maintained only until Day 7. Media is partially changed on Days 7, 14, 21, and 28 to support neuronal maturation.



This is based on Abud *et al*, 2017 protocol with minor modifications. The process begins with the differentiation of iPSCs into hematopoietic progenitors using a chemically defined medium supplemented with factors such as FGF2, BMP4, Activin A, VEGF-A, SCF, IL-3, and IL-6. By day 6,floating cells are collected and put back in culture after each media change and cultured in a microglia differentiation medium containing CSF1, IL-34, and TGF-β. In the final 3 days, the medium is further supplemented with CD200 and CX3CL1 to promote maturation.

Load test: data were acquired in either data-independent acquisition (DIA, left side) or data-dependent acquisition (DDA, right side) or methods with 35 min gradients. The total number of protein groups ID'ed in each sample are marked on top of each bar. We tested various MS parameters (e.g., gradient length, column length, on-column digested protein load amount, etc.) on our Orbitrap Exploris 480. Shorter gradients and higher load (e.g, 1ng on-column) provided higher protein ID numbers irrespective of traditional DDA (>2700 IDs) approach or the more current popular DIA (>3000 IDs) approach compared to getting ~1000 or ~1500 IDs, respectively, with 500pg on column protein load. Proteins identified with at least two peptides were considered.



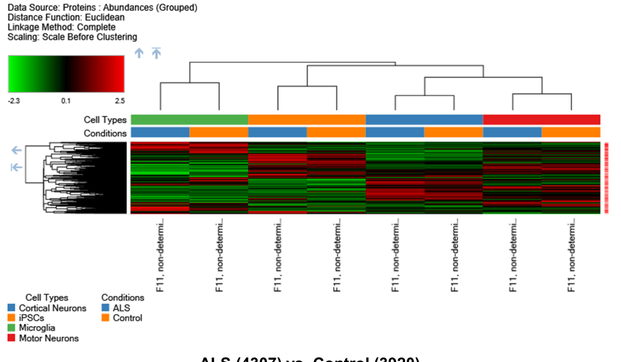


Results

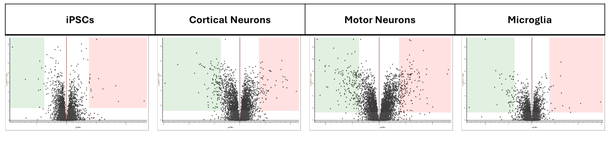
Sample set	MS2/MS3 quantification*	High-confidence protein identifications
TMTpro 16-plex • *Synaptosome + brain homogenate samples* • *7 offline fractions*	MS2	6,067
TMTpro 18-plex • *Synaptosome samples* • *9 offline fractions*	MS3	4,301
TMTpro 18-plex • *Brain homogenate samples* • *9 offline fractions*	MS3	2,905

*MS2-based quantification provides more protein identifications; MS3-based quantification provides better quantitative accuracy

Reporter Ion	Sample Name
126	iPSCs-3920
127N	iPSCs-3920
127C	iPSCs-4307
128N	iPSCs-4307
128C	Cortical Neurons-3920
129N	Cortical Neurons-3920
129C	Cortical Neurons-4307
130N	Cortical Neurons-4307
130C	Motor Neurons-3920
131N	Motor Neurons-3920
131C	Motor Neurons-4307
132N	Motor Neurons-4307
132C	Microglia-3920
133N	Microglia-3920
133C	Microglia-4307
134N	Microglia-4307



ALS (4307) vs. Control (3920)



	Cortical Neurons Comparison		iPSCs Comparison		Microglia Comparison		Motor Neurons Comparison	
Proteins Up/Down Regulated	9 ↑	14↓	108↑	56↓	154↑	160↓	45↑	15↓
# of significant proteins (p<0.05)	547	365	1073	1266	1409	1512	597	580
# of proteins log_2FC <+/-0.75	13	29	118	69	163	176	57	18

Acknowledgments

The authors declare no competing financial interest.

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