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14. ABSTRACT The limited access to patient neurons greatly impedes the progress of research in dystonia. The major purpose of this project is to develop a novel cellular model system for dystonia research. We have successfully generated human patient-specific neurons through either direct conversion of patients' skin fibroblasts or differentiation of induced pluripotent stem cells (iPSCs). We found that dystonia neurons show developmental delay, thickened nuclear lamina, disrupted nuclear morphology, and impaired nucleocytoplasmic transport. Furthermore, we have uncovered that nuclear lamina protein LMNB1 is upregulated in DYT1 neurons and exhibits abnormal subcellular distribution in a cholinergic motor neuron (MN)-specific manner. Interestingly, downregulation of LMNB1 can largely ameliorate all the cellular defects in DYT1 MNs. These results reveal high value of disease modeling with human patient-specific neurons and indicate that dysregulation of nuclear LMNB1 may constitute a major molecular mechanism underlying DYT1 pathology.					
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1. INTRODUCTION:

Dystonia is the third most common movement disorder and characterized by sustained or intermittent muscle contractions, leading to abnormal movements and postures. Veterans are particularly at risk due to the frequent occurrence after head trauma and potentially irreversible side effects of medications used to treat PTSD (post-traumatic stress disorder). Because the clinical characteristics and underlying causes are very heterogeneous, the pathological mechanisms of dystonia remain largely unknown and the diagnosis and etiological definition of this disorder remain a challenge. However, the limited access to patient neurons greatly impedes the progress of research in dystonia. The major purpose of this project is to develop a novel cellular model system for dystonia research and examine the cellular and molecular pathogenesis of this disease. We will generate human patient-specific neurons through either direct conversion of patients' skin fibroblasts or differentiation of induced pluripotent stem cells (iPSCs). These patient-specific neurons will be extensively examined and compared with healthy control cells at cellular morphology, neurodevelopment, survival, nuclear transport and functions. Expected results emanating from this study will provide novel insights into dystonia pathology and potentially lead to molecular targets for therapeutic interventions.

2. KEYWORDS:

Dystonia, Human Neurons, Motor Neurons, Reprogramming, TOR1A

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Major goals:

1. To establish the protocol of directly reprogramming human neurons from adult fibroblasts.

Milestones achieved:

1.1 Obtained all research materials, including fibroblast cell lines are used in this project.
100% completion.

1.2 Established the protocol of reprogramming human neurons from adult fibroblasts.
100% completion. We are able to generate patient-specific motor neurons.

1.3 Verified the neuronal identity, electrophysiology and maturation of reprogrammed neurons.
50% completion. Electrophysiological analysis needs to be done.

1.4 Successfully generated dystonia neurons.
100% completion. Other neuronal subtypes will be generate, including from adulthood-onset patients.

2. To examine the nuclear morphology and nucleocytoplasmic transport in dystonia neurons.

Milestones Achieved:

2.1 Optimized conditions of immune-staining reprogrammed neurons.
100% completion.

2.2 Acquired plenty of confocal micrographs for data analysis.
50% completion. More target proteins will be analyzed.

2.3 Optimized protocols for EM sample preparation.
100% completion.

2.4 Acquired plenty of EM data of reprogrammed neurons.
50% completion. More EM samples will be prepared and examined.

3. To examine nucleocytoplasmic transport (NCT) in dystonia neurons.

Milestones Achieved:

3.1 Optimized Fluorescence in situ hybridization (FISH) condition for nuclear mRNA export assay in reprogrammed neurons.
100% completion.

3.2 Optimized condition for dual reporter assay.
100% completion.

3.3 Acquired plenty of confocal micrographs of FISH data and dual reporter data.
100% completion.

3.4 Quantified the micrographs by ImageJ.
80% completion. More samples will be analyzed.

3.5 Organized data and prepared manuscript (s).
During project year 2022-2023, this project has generated four peer-reviewed papers so far, including 3 research papers and 1 invited review. (See appendix A-D)

What was accomplished under these goals?

During this reporting period (March 15, 2022, to March 14, 2023), this project achieved the following accomplishments.

1. We have generated and characterized the induced pluripotent stem cells (hiPSC) by genome editing for modeling DYT1 dystonia.

We have generated two sets of DYT1 dystonia iPSC lines, including a GAG deletion in the *TOR1A* gene from WT-iPSCs and a GAG knock-in to correct patient iPSCs. In the first set, we identified two clones of heterozygous and one clone of homozygous GAG deletion as well as two wild-type isogenic controls. In the correction set, we obtained two clones of corrected lines, in which GAG deletions have been restored. All clones were verified by DNA sequencing. The pluripotency of these iPSC lines was further verified by Alkaline Phosphatase (AP) staining, the highly expressed pluripotency markers, and in vitro differentiation of iPSCs to embryoid bodies (EBs), which highly expressed trilineage markers. Using these dystonia iPSCs, we could generate a large number of patient-specific neurons to decipher the pathogenesis of the disease via biochemical approaches. The information about generation and characterization of these hiPSC lines has been published in *Stem Cell Research* (See Appendix A).

2. We have established the protocol for generation of highly pure motor neurons from hiPSCs

We developed the protocol of generation of highly pure motor neurons from hiPSCs. Using this protocol, we have successfully generated large amount of motor neurons from DYT1 iPSC lines and isogenic controls, providing invaluable research materials to determine the pathogenesis of DYT1 dystonia. This protocol has been published in *Star Protocols* (See Appendix B) and *Cells* as a review (See Appendix C).

3. We have developed a protocol to image and quantify nucleocytoplasmic transport in cultured cells using fluorescent in situ hybridization and a dual reporter system.

Using this protocol, we are able to measure the nucleocytoplasmic transport (NCT) of both mRNA and protein cargos at a single cell level. This protocol was published in *Star Protocols* (See Appendix D).

4. Presented our work on international conferences as an invited speaker.

I presented our work “Patient-specific neurons are key to new discoveries in neurological diseases” on The Global Experts Meet on Neurology, Neuroscience and Brain Disorders, which was held on April 27-29, 2023. Paris, France. (Invited Speaker, In Person). (See Appendix E).

What opportunities for training and professional development has the project provided?

During this reporting period, this project provided 2 Graduate Research Assistant (GRA) positions (person semester) and research opportunity for 1 high school summer researcher. Through participating this project, trainees learned the basic skills of cell culture, immunostaining, imaging and the advanced techniques of reprogramming human neurons from adult fibroblasts.

The PI attended three conferences and presented the new findings of this project.

1. April 27, 2023. *Patient-specific neurons are key to new discoveries in neurological diseases*. The Global Experts meet on Neurology, Neuroscience and Brain Disorders. April 27-29, 2023. Paris, France. (Invited Speaker, In Person) (See Appendix E).
2. June 15, 2022. *Modeling Neurological Diseases Using Patient-derived Neurons*. The 5th Edition of International Conference on Neurology and Neurological Diseases. June 15-16, 2022. (Invited Speaker. Virtual) (See Appendix F).
3. December 4, 2022. **Ding B.** *Impaired Nucleocytoplasmic Transport in Neurological Diseases*. American Society for Cell Biology (ASCB) Annual Meeting. Washington DC. December 3-7, 2022. (In person) (See Appendix G).

How were the results disseminated to communities of interest?

The new findings from this project were published in prestigious peer-reviewed journals and the PI also presented the new findings to scientific communities in scientific conferences. (See appendix A-G)

What do you plan to do during the next reporting period to accomplish the goals?

1. Generate more human neurons from dystonia patients and healthy controls.

Using both direct conversion and iPSC-based approach to generate different neuronal subtypes, including motor neurons, cholinergic interneurons and generic neurons.

2. Examine the cellular defects in DYT1 neurons.

We will complete the examination of the nuclear morphology of DYT1 neurons by TEM, and analyze the nuclear lamina and nuclear pore complex.

3. Organize the data and prepare more manuscripts.

The new findings will be organized and published in peer-reviewed journals.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

We have developed and further optimized the techniques for generation of human neurons from patient skin fibroblasts. These techniques overcome the limited access to human brain neurons and provide valuable materials in dystonia research. Using patient-derived neurons, we reported the disease-dependent cellular deficits, including dysregulation of nuclear protein LMNB1 and impaired nucleocytoplasmic transport. When we used a technique to bring down the LMNB1 protein level and the cellular defects in dystonia neurons were corrected. These findings reveal high value of disease modeling with human patient-specific neurons and indicate that dysregulation of nuclear LMNB1 may constitute a major molecular mechanism underlying DYT1 dystonia pathology. Downregulation of LMNB1 could be a novel target for developing therapeutic interventions in DYT1 dystonia.

Additionally, we have generated two sets of iPSC lines for modeling DYT1 dystonia, providing valuable research materials in dystonia research. The protocols we have developed for the generation of patient-specific neurons and nuclear transport analysis has been shared within the research filed as open access publications.

What was the impact on other disciplines?

Besides the impact on neuroscience and disease modeling, the findings from this project have a broad impact on other disciplines. For example, the regulations of nucleocytoplasmic transport involves the Cell Biology, Molecular Biology and Biochemistry. Dysregulation of LMNB1 in dystonia neurons advanced our understanding of the impact of nuclear lamina on nuclear envelope morphology and nucleocytoplasmic transport.

What was the impact on technology transfer?

The findings from this project reveal high value of disease modeling with human patient-specific neurons and indicate that dysregulation of nuclear LMNB1 may constitute a major molecular mechanism underlying DYT1 dystonia pathology. Downregulation of LMNB1 could be a novel target for developing therapeutic interventions in DYT1 dystonia.

What was the impact on society beyond science and technology?

Nothing to Report

5. CHANGES/PROBLEMS:

Nothing to Report

Changes in approach and reasons for change

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them

Because of the crisis of Covid-19 and lab relocation, the research and the expenditure of the fund was significantly delayed.
In January 2022, the PI and his team relocated to LSU Health Shreveport, where excellent research core facilities and neuroscientists are available on campus. The new lab has been established and the research activities are fully resumed. The strong support at the new institution will significantly facilitate the proposed studies of this project. We aim to complete all proposed work of this project by March 14, 2024.

Changes that had a significant impact on expenditures

The pandemic of Covid-19 has a significant impact on the expenditures. During university closure, we had to stop or minimize our research activities and collaborations. After university reopen, the lab was only partly functional. The overall expenditure during this reporting period was significantly delayed. Additionally, the PI and his team relocated to LSU Health Shreveport in early 2022. The relocation also delayed the expenditure.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Nothing to Report

Significant changes in use or care of vertebrate animals

Nothing to Report

Significant changes in use of biohazards and/or select agents

Nothing to Report

6. PRODUCTS:

- **Publications, conference papers, and presentations**

- Journal publications.**

1. Akter M, Cui H, Chen Y-H and **Ding B***. (2022). Generation of gene-corrected isogenic control cell lines from a DYT1 dystonia patient iPSC line carrying a heterozygous GAG mutation in TOR1A gene. **Stem Cell Research**. 62.July 2022, 102807. (See Appendix A). *Published, acknowledgement of federal support (yes).*
2. Akter M, Cui H, Sepehrimanesh M., Hosain A. and **Ding B***. (2022). Generation of highly pure motor neurons from human induced pluripotent stem cells. **STAR Protoc**. 2022 Mar 10; 3(1):101223. (Invited protocol. Cell Press) (See Appendix B). *Published, acknowledgement of federal support (yes).*
3. Akter M and **Ding B***. (2022). Modeling Movement Disorders via Generation of hiPSC-Derived Motor Neurons. **Cells**. 2022 Nov 27;11(23):3796. (See Appendix C). *Published, acknowledgement of federal support (yes).*
4. Cui H, Sepehrimanesh M, Coutee C, Akter M, Hosain A and **Ding B***. (2022). Protocol to image and quantify nucleocytoplasmic transport in cultured cells using fluorescent in situ hybridization and a dual reporter system. **STAR Protoc**. 2022 Dec 16; 3(4):101813. (See Appendix D). *Published, acknowledgement of federal support (yes).*

Nothing to Report

Other publications, conference papers and presentations.

Presentation:

The PI attended three conferences and presented the new findings of this project.

1. April 27, 2023. *Patient-specific neurons are key to new discoveries in neurological diseases*. The Global Experts meet on Neurology, Neuroscience and Brain Disorders. April 27-29, 2023. Paris, France. (Invited Speaker, In Person) (*See Appendix E*).
2. June 15, 2022. *Modeling Neurological Diseases Using Patient-derived Neurons*. The 5th Edition of International Conference on Neurology and Neurological Diseases. June 15-16, 2022. (Invited Speaker. Virtual) (*See Appendix F*).
3. December 4, 2022. **Ding B.** *Impaired Nucleocytoplasmic Transport in Neurological Diseases*. American Society for Cell Biology (ASCB) Annual Meeting. Washington DC. December 3-7, 2022. (In person) (*See Appendix G*).

- **Website(s) or other Internet site(s)**

Nothing to Report

- **Technologies or techniques**

Developed and optimized protocols for generation human neurons from adult fibroblasts and hiPSC, and the techniques for measuring nuclear transport in cultured neurons. These technologies have been shared via publications in peer-reviewed journals (*see appendix B, C and D*).

- **Inventions, patent applications, and/or licenses**

Nothing to Report

- **Other Products**

Research materials:

We have generated two sets of DYT1 dystonia iPSC lines. The information about the generation and characterization of these cell lines has been published as a lab resource paper in *Stem Cell Research* (*see Appendix A*).

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name	Baojin Ding	Masuma Akter	Haochen Cui	Masood Sepehrmanesh
Project role	PI	Graduate Student	Graduate Student	Postdoc
ORCID ID	0000-0002-2149-2599	N/A	N/A	0000-0002-6300-2906
Nearest person month worked	4	5	5	3
Contribution to Project	Supervision for the project, including design experiment, train the graduate students and prepare manuscripts and presentations for scientific meetings.	Performed experiments, including culture cells, reprogramming human neurons, and neuronal survival assay.	Performed experiments, including culture cells, plasmid purification, lentivirus preparation.	Performed experiments, including culture cells and lentivirus preparation. Helped PI oversee the lab.
Funding support	Start-up and NIH	University fellowship	Fellowship from LSU Health Shreveport. this Award	Start-up and NIH

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Title: Biochemically identify dysregulated factors in DYT1 dystonia via iPSC-derived neurons
Agency: Center for Brain Health, LSU Health Shreveport
Type: Research Grant
Grant Number: CBH Grant-In-Aid Spring 2022
Role: Principal Investigator
Dates: 07/01/2022 - 12/30/2023
Total amount:
Overall goal: This intramural grant aims to help generate more preliminary data for applying extramural awards.

What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

Nothing to Report

QUAD CHARTS:

Nothing to Report

9. APPENDICES:

Appendix A-Journal article

Akter M, Cui H, Chen Y-H and **Ding B***. (2022). Generation of gene-corrected isogenic control cell lines from a DYT1 dystonia patient iPSC line carrying a heterozygous GAG mutation in TOR1A gene. **Stem Cell Research**. 62.July 2022, 102807.

Appendix B-Journal article

Akter M, Cui H, Sepehrimanesh M., Hosain A. and **Ding B***. (2022). Generation of highly pure motor neurons from human induced pluripotent stem cells. **STAR Protoc**. 2022 Mar 10; 3(1):101223.

Appendix C-Journal article

Akter M and **Ding B***. (2022). Modeling Movement Disorders via Generation of hiPSC-Derived Motor Neurons. **Cells**. 2022 Nov 27;11(23):3796.

Appendix D-Journal article

Cui H, Sepehrimanesh M, Coutee C, Akter M, Hosain A and **Ding B***. (2022). Protocol to image and quantify nucleocytoplasmic transport in cultured cells using fluorescent in situ hybridization and a dual reporter system. **STAR Protoc**. 2022 Dec 16; 3(4):101813.

Appendix E- Abstract-Oral presentation

April 27, 2023. *Patient-specific neurons are key to new discoveries in neurological diseases*. The Global Experts meet on Neurology, Neuroscience and Brain Disorders. April 27-29, 2023. Paris, France. (Invited Speaker, In Person)

Appendix F- Abstract- Oral presentation

June 15, 2022. *Modeling Neurological Diseases Using Patient-derived Neurons*. The 5th Edition of International Conference on Neurology and Neurological Diseases. June 15-16, 2022. (Invited Speaker. Virtual)

Appendix G- Abstract-Poster presentation

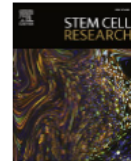
December 4, 2022. **Ding B.** *Impaired Nucleocytoplasmic Transport in Neurological Diseases*. American Society for Cell Biology (ASCB) Annual Meeting. Washington DC. December 3-7, 2022. (In person) (*See Appendix G*).

Appendix H-the PI's updated CV



Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr

Lab Resource: Multiple Cell Lines



Generation of gene-corrected isogenic control cell lines from a DYT1 dystonia patient iPSC line carrying a heterozygous GAG mutation in *TOR1A* gene

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ABSTRACT

Childhood-onset torsion dystonia (DYT1) is a rare hereditary movement disorder and usually caused by a heterozygous GAG deletion (c.907–909) in the *TOR1A* gene (ΔE , p.Glu303del). The neuronal functions of torsin proteins and the pathogenesis of ΔE mutation are not clear. Previously, we have generated a hiPSC line from DYT1 patient fibroblast cells. In this study, we genetically corrected GAG deletion and obtained two isogenic control lines. These hiPSC lines contain the wild-type *TOR1A* sequence, showed the normal stem cell morphology and karyotype, expressed pluripotency markers, and differentiated into three germ layers, providing a valuable resource in DYT1 research.

1. Resource Table

Unique stem cell lines identifier	LSUHSi003-A-2 LSUHSi003-A-3
Alternative names of stem cell lines	DYT1-CR-4B2 (CSUi002-A-2) DYT1-CR-A6 (CSUi002-A-3)
Institution	Louisiana State University Health Sciences Center in Shreveport, LA USA
Contact information of the reported cell line distributor	Baojin Ding (baojin.ding@lsuhs.edu)
Type of cell lines	iPSC
Origin	Human
Additional origin info (Applicable for human ESC or iPSC)	Age: 30 YR Sex: Male Ethnicity: White
Cell Source	hiPSC (CSUi002-A)
Method of reprogramming	N/A
Clonality	Clonal
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	RT/q-PCR
Cell culture system used	Serum-free and feeder-free medium
Type of Genetic Modification	Gene correction of pathogenic mutation
Associated disease	DYT1 dystonia
Gene/locus	<i>TOR1A</i> c.907_909delGAG (p.Glu303del)/9q34.11
Multiline rationale	Isogenic clones with corrected GAG mutation

(continued on next column)

(continued)

Unique stem cell lines identifier	LSUHSi003-A-2 LSUHSi003-A-3
Method of modification/site specific nuclease used	CRISPR/Cas9
Site-specific nuclease (SSN) delivery method	Electroporated with a 4D-Nucleofector (Lonza) using CA-137 program.
All genetic material introduced into the cells	Synthetic gRNA (IDT)
Analysis of the nuclease-targeted allele status	HiFi Cas9 nuclease V3 (IDT)
Method of the off-target nuclease activity surveillance	Sequencing of the targeted allele
Name of transgene	Targeted PCR/sequencing
Eukaryotic selective agent resistance (including inducible/gene expressing cell-specific)	N/A
Inducible/constitutive system details	N/A
Date archived/stock date	January 21, 2022
Cell line repository/bank	https://hpscrgen.eu/cell-line/LSUHSi003-A-2 https://hpscrgen.eu/cell-line/LSUHSi003-A-3
Ethical/GMO work approvals	Genetic modification was performed at Genome Engineering and iPSC Center (GEIC) at Washington University in St. Louis.

(continued on next page)

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(continued)

Unique stem cell lines identifier	LSUHSi003-A-2 LSUHSi003-A-3
Addgene/public access repository recombinant DNA sources/ disclaimers (if applicable)	N/A

2. Resource utility

The patient derived DYT1 hiPSCs together with these genetically corrected isogenic controls will provide invaluable resources for DYT1 dystonia research. Neurons derived from hiPSCs will enable us to biochemically identify dysregulated factors in diseased neurons and potentially discover novel molecular targets for therapeutic interventions (Table 1).

3. Resource details

DYT1 dystonia is a movement disorder mainly caused by a heterozygous trinucleotide deletion (Δ GAG; c.907–909) in the *TOR1A* gene and manifests as sustained or intermittent muscle contractions (Ding et al., 2021). It is often initiated or worsened by voluntary action and associated with overflow muscle activation. The specific mechanism by which the *TOR1A* mutation leads to the development of dystonic phenotype is still unknown. Even though animal models provide insights into disease mechanisms, significant species-dependent differences exist because animals with identical heterozygous mutation (Δ E) fail to show the pathology seen in human patients (Goodchild et al., 2005). In addition, the limited access to patient neurons greatly impedes the progress of research in dystonia. Excitingly, the generation of patient-specific neurons via human induced pluripotent stem cells (hiPSCs) provides an unprecedented approach for dystonia research (Akter et al., 2021; Akter et al., 2022). Previously, we generated a hiPSC line (CSUi002-A) from a DYT1 patient fibroblast cell line that is carrying a heterozygous mutation in the *TOR1A* gene (Ding et al., 2021; Wu et al., 2021). In this study, we genetically corrected the GAG deletion in this line with CRISPR/Cas9 method and obtained two hiPSC lines (LSUHSi003-A-2 and LSUHSi003-A-3), in which the GAG deletion in the *TOR1A* gene was restored. These isogenic controls together with the DYT1 patient hiPSC line provide a valuable resource in DYT1 dystonia research.

Both mutation-corrected hiPSC lines showed a typical pluripotent stem cell morphology with a high nucleus/cytoplasm ratio (Fig. 1A). DNA sequencing confirmed that the GAG deletion was restored in both newly generated hiPSC lines (LSUHSi003-A-2 and LSUHSi003-A-3) (Supp. Fig.S1A and B). Sanger DNA sequencing of polymerase chain reaction (PCR) products further verified that the DYT1 iPSC line (CSUi002-A) contains the heterozygous GAG deletion (c.907–909) in the *TOR1A* gene (one copy is wild-type and the sequence of the other copy is shifted due to GAG deletion), and this deletion was corrected in both newly generated lines (Fig. 1B). GTW banding method was used to analyze the chromosomes from both corrected hiPSC lines and demonstrated that they are normal male karyotype, 46, XY (Fig. 1C). Short tandem repeat (STR) analysis at 15 loci identified a 100% allele match between DYT1 and DYT1 correction clones (Supp. Fig.S1C). Immunostaining indicated that these iPSCs highly expressed pluripotency markers of *OCT4*, *NANOG*, *SOX2*, and *SSEA4* (Fig. 1D). Quantitative RT-PCR analysis demonstrated that the pluripotency markers of *OCT4*, *SOX2*, *NANOG*, and *KLF4* in DYT1 correction lines were expressed at similar levels as the parental line (CSUi002-A) (Fig. 1E). Following spontaneous differentiation, embryoid bodies (EBs) (Fig. 1F) derived from both corrected cell lines displayed dramatic upregulation of markers of the ectoderm (*PAX6*, *OTX1*), mesoderm (*DCN*, *IGF2*, *GATA2*), and endoderm (*SOX7*, *SOX17*) lineages. The expression levels of these

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Brightfield microscopy	Typical primed pluripotent human stem cell morphology	Fig. 1 Panel A
Pluripotency status evidence for the described cell line	Qualitative analysis	Immunocytochemistry showed expression of pluripotency markers: <i>OCT4</i> , <i>SOX2</i> , <i>NANOG</i> , <i>SSEA4</i>	Fig. 1 Panel D
	Quantitative analysis	Compared to DAPI, % of positive cell (LSUHSi003-A-2, LSUHSi003-A-3) <i>OCT4</i> : 97%, 96%; <i>SOX2</i> : 98%, 97%; <i>NANOG</i> : 95%, 96%; <i>SSEA4</i> : 96%, 97%. RT-PCR showed highly express <i>OCT4</i> , <i>SOX2</i> , <i>NANOG</i> , <i>KLF4</i>	Fig. 1 Panel D and E
Karyotype	Karyotype (G-banding) and resolution	46, XY, Resolution 400	Fig. 1 Panel C
Genotyping for the desired genomic alteration/ allelic status of the gene of interest	PCR across the edited site and deep sequencing analysis	Heterozygous GAG deletion (c.970–909) in <i>TOR1A</i> gene was restored.	Fig. 1 Panel B and Supplementary Fig. S1A and B
	Transgene-specific PCR	N/A	N/A
Verification of the absence of random plasmid integration events	PCR/Southern	Off Target Analysis of gRNA showed 100% minus a weighted sum of off target hit-scores in the target genome.	N/A
Parental and modified cell line genetic identity evidence	STR analysis, microsatellite PCR (mPCR) or specific (mutant) allele seq	STR analysis of 15 loci, all matched.	Supplementary Fig. S1C
Mutagenesis / genetic modification outcome analysis	Sequencing (genomic DNA PCR or RT-PCR product)	The sequencing results of genomic DNA all matched with parent line.	Fig. 1 Panel B and Supplementary Fig. S1A and B
	PCR-based analyses	The sequencing results PCR products all matched with parent line.	Fig. 1 Panel B and Supplementary Fig. S1A and B
Off-target nuclease analysis	Southern Blot or WGS; western blotting (for knockouts, KOe)	N/A	N/A
	PCR across top 5/10 predicted top likely off target sites, whole genome/exome sequencing	N/A	N/A
Specific pathogen-free status	Mycoplasma	Tested by MycoAlert PLUS kit: Negative	Fig. S1D
Multilineage differentiation potential	Embryoid body formation, RT-PCR	Upregulation of trilineage markers <i>PAX6</i> , <i>OTX1</i> (ectoderm), <i>DCN</i> , <i>IGF2</i> , <i>GATA2</i> (mesoderm), and	Fig. 1 Panel F and G

(continued on next page)

Table 1 (continued)

Classification	Test	Result	Data
Donor screening (OPTIONAL)	HIV 1 + 2	SOX7, SOX17 (endoderm).	N/A
	Hepatitis B, Hepatitis C	N/A	
Genotype additional info (OPTIONAL)	Blood group	N/A	N/A
	HLA tissue typing	N/A	

trilineage markers were consistent with the parental line (CSUi002-A) and much higher than undifferentiated iPSCs (Fig. 1G). PCR screening demonstrated that both DYT1 correction hiPSC lines (LSUHSi003-A-2 and LSUHSi003-A-3) were negative for mycoplasma (Supp. Fig.S1 D).

4. Materials and methods

4.1. Correction and culture of DYT1 iPSCs

The GAG mutation in the *TOR1A* gene in DYT1 hiPSC was genetically corrected at the Genome Engineering and iPSC Center (GEIC) at Washington University in St. Louis. Briefly, approximately 1×10^6 single cells were resuspended in P3 primary buffer (Lonza) with gRNA/Cas9 ribonuclease protein (RNP) complex (200 pmol synthetic gRNA and 80 pmol HiFi Cas9 protein) and *TOR1A* correction ssODN (Table 2). A silent mutation (C to T) was introduced in the donor oligo sequence (ssODN) to avoid the re-cutting of the edited sequence by CRISPR/Cas9. Subsequently, cells were electroporated with a 4DNucleofector (Lonza) using the CA-137 program. Following nucleofection, the editing efficiency was confirmed by targeted deep sequencing using primer sets specific to target regions and then the pool was single-cell sorted. Single cell clones were screened with targeted deep sequencing analysis. All iPSCs were cultured with mTeSR Plus (STEMCELL Technology) on Matrigel-coated plates at 37 °C in a humidified, 5% CO₂ incubator and passage at a 1:6

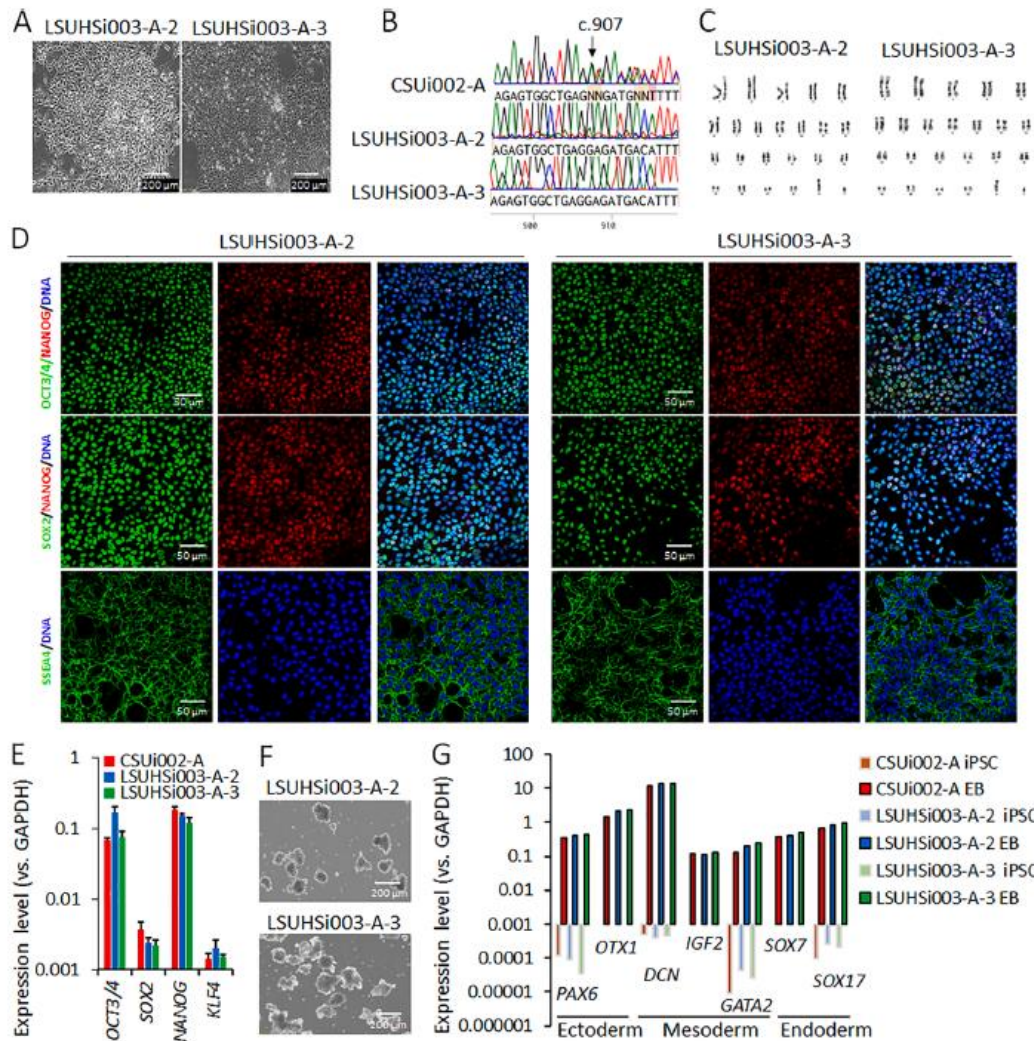


Fig. 1. Characterization of DYT1 correction iPSC lines.

Table 2
Reagents details.

Antibodies used for Immunocytochemistry		Dilution	Company Cat # and RRID
	Antibody		
Pluripotency Markers	Mouse anti-OCT4	1:200	Santa Cruz Cat# sc-5279, RRID: AB_628051
	Mouse anti-SOX2	1:200	Santa Cruz Biotechnology Cat# sc-365823, RRID: AB_10042165
	Mouse anti-SSEA4	1:200	Abcam Cat# ab16287, RRID: AB_778073
	Rabbit anti-Nanog	1:100	Abcam Cat# ab21624, RRID: AB_446437
Secondary antibodies	Donkey anti-Mouse IgG (H + L), Alexa Fluor 488	1:500	Jackson ImmunoResearch Labs Cat# 715-545150, RRID: AB_2340846
	Donkey Anti-Rabbit IgG (H + L), Alexa Fluor 594	1:500	Jackson ImmunoResearch Labs Cat# 711-585152, RRID: AB_2340621
Nuclear stain	Hoechst33342	1 µg/mL	Invitrogen Cat # H3570, RRID: NOT FOUND
Site-specific nuclease			
Nuclease information	HiFi Cas9 nuclease	HiFi Cas9 nuclease (IDT, Cat #1081061)	
Delivery method	electroporation	4D-Nucleofector (Lonza, Cat # AAF-1002B)	
Selection/enrichment strategy	sorted into 96-well plates with one cell per well	Single cell clones were screened and expanded	
Primers and Oligonucleotides used in this study			
	Target	Forward /Reverse primer (5'-3')	
Pluripotency marker	OCT 3/4	CGAGAGGATTTTGGAGGCTGC/ CGAGGAGTACAGTGCACTGA	
Pluripotency marker	SOX 2	AGGATAAGTACACGCTGCCC/ TTTCATGTGCGGTAACTGTC	
Pluripotency marker	NANOG	TGCTCTCTGCTGAGATGCCT/ CAGAAAGTGGGTGTTTGCT	
Pluripotency marker	KLF4	TCTCCAATTGCTGACCCAT/ CGGATCGGATAGGTGAAGCT	
Differentiation marker	PAX6	GGGCGGAGTTATGATACCTACA/ ATATCAGGTTCACTTCGGGAA	
Differentiation marker	OTX1	TACGCCCTCTCTTCTACT/ GCATGTGGGTGGTGATGATG	
Differentiation marker	DCN	CTGAAGAACCCTTCAGCATTGA/ GGCAATTCCTTCAGCTGATTCT	
Differentiation marker	IGF2	CAATATGACACCTGGAAGCAGT/ GTAGAGCAATCAGGGGACGG	
Differentiation marker	GATA2	ACCTGTTGTGCAAAATTGTCAGA/ ATCCCTTCCTTCTCATGTGCA	
Differentiation marker	SOX7	ACTCCACTCCAACTCCAAAG/ TTCATTGGGATCCATGTCCC	
Differentiation marker	SOX17	ATCGGGGACATGAAGGTGAA/ TCCTTAGCCACACCATGAA	
Housekeeping	GAPDH	CAAATTCATGGCACCGTCA/ GGACTCCACGACGTACTCAG	
Genes		ACAGCAGCTTAATTGACCGGA/ ATCATGAGCCCTGCGATGAG	
Genotyping-PCR	TOR1A	GTGTATCCGAGTGGAAATGC TGAAGACATTGTAAGCAGAG	
Sequencing	TOR1A	AATGTGTATCCGAGTGGAAATGCAGTCCCGAGGCTATGAAATTGATGAA	
TOR1A gRNA (IDT)	TOR1A	GACATTGTAAAGTAGAGTGGCTGAGGAGATGACATTTTCCCAAAGAGGAGA	
TOR1A correction ssODN (IDT)	TOR1A	GAGTTTCTCAGATAAAGGCTGCA	

ratio using gentle cell dissociation reagent (Versene, Gibco).

4.2. Embryoid bodies (EB) formation

As our previous report (Akter et al., 2021), cultured hiPSCs were dissociated with Versene and transferred to low attachment 10-cm petri dishes in KOSR medium (DMEM/F12 medium containing 20% KnockOut Serum Replacement, 1% GlutaMax, 1% non-essential amino acids, 50 µM β-mercaptoethanol and 1% penicillin–streptomycin) in the presence of 10 µM Y-27632 (STEMCELL Technologies). Changed the medium every other day and EBs gradually formed (Fig. 1F). After 7 days of suspension culture, EBs were digested with 0.25% Trypsin and cultured on gelatin coated plates with KOSR medium for another 7 days. The total RNAs were extracted for RT-PCR analysis of trilineage markers.

4.3. Immunostaining and confocal microscopy

Cultured iPSCs were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature and incubated in blocking buffer (3% bovine serum albumin in PBS) with (for nuclear markers) or without (for a cell surface marker SSEA4) 0.2% Triton X-100 for 1 h. Cells were then sequentially incubated with primary and secondary antibodies (Table 2)

as previously described (Akter et al., 2021). Hoechst 33,342 (Invitrogen) was used to stain nuclei. Images were obtained with a Leica SP5 confocal microscope.

4.4. Quantitative PCR analysis

As previously report (Akter et al., 2021), cultured iPSCs and EBs were collected and lysed in TRIzol (Invitrogen). Total RNAs were extracted using the phenol/chloroform method, and then reverse-transcribed into cDNAs using the SuperScript™ III Reverse Transcriptase (Invitrogen). Quantitative PCR analysis was performed using SYBR Green PCR Master Mix (Applied Biosystems) and run on a StepOne qPCR machine (Applied Biosystems). The gene expression data were analyzed using the $\Delta\Delta C_T$ method and the values were normalized to the expression of the housekeeping gene GAPDH (Fig. 1E and G). Primers used in this study were listed in Table 2.

4.5. Karyotyping

Chromosomes from iPSC clones were analyzed using the GTW banding method at GEiC at Washington University in St. Louis.

4.6. STR analysis

Short tandem repeat (STR) analysis of 15 loci (Fig. S1C) were performed at GEiC at Washington University in St. Louis.

4.7. Mycoplasma test

Mycoplasma test was performed by MycoAlert PLUS kit (Lonza) at GEiC at Washington University in St. Louis (Fig. S1D).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scr.2022.102807>.

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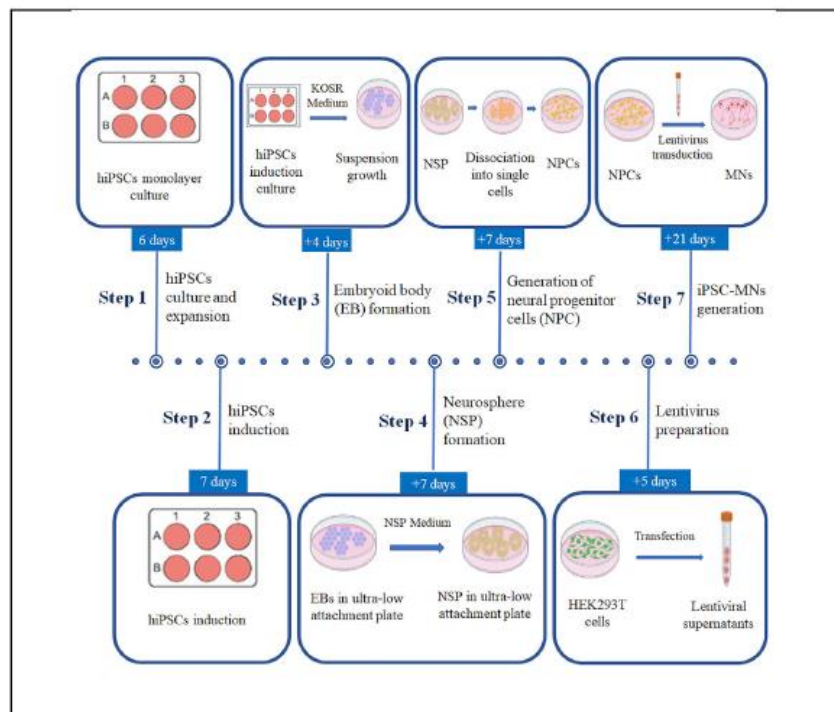
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Protocol

Generation of highly pure motor neurons from human induced pluripotent stem cells



Generation of human motor neurons (MNs) overcomes the inaccessibility to patient brain tissues and greatly facilitates the research in MN-related diseases. Here, we describe a protocol for generation of neural progenitor cells (NPCs) from human induced pluripotent stem cells (hiPSCs), followed by preparation of functional MNs. The optimized induction condition with the expression of three transcription factors in a single lentiviral vector significantly improved the yield and purity, making it possible to biochemically identify dysregulated factors in diseased neurons.

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Highlights
Detailed protocol to
generate motor
neurons from human
induced pluripotent
stem cells

Protocol assesses the
identity and efficiency
of motor neuron
generation *in vitro*

Optimized induction
condition improves
the yield and purity of
motor neurons

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Protocol

Generation of highly pure motor neurons from human induced pluripotent stem cells

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<https://doi.org/10.1016/j.xpro.2022.101223>

SUMMARY

Generation of human motor neurons (MNs) overcomes the inaccessibility to patient brain tissues and greatly facilitates the research in MN-related diseases. Here, we describe a protocol for generation of neural progenitor cells (NPCs) from human induced pluripotent stem cells (hiPSCs), followed by preparation of functional MNs. The optimized induction condition with the expression of three transcription factors in a single lentiviral vector significantly improved the yield and purity, making it possible to biochemically identify dysregulated factors in diseased neurons. For complete details on the use and execution of this protocol, please refer to Ding (2021), Ding et al. (2021), and Sepehrimanesh and Ding (2020).

BEFORE YOU BEGIN

1. Obtain the permission from institutional animal care and use committee (IACUC) for the animal study. This protocol has been approved by IACUC at University of Louisiana at Lafayette.
2. Order the antibodies and qPCR primers that are listed in the [key resources table](#) for quality control and assessment of conversion efficiency.
3. Acquire all critical reagents mentioned in the [key resources table](#).
4. Acquire/thaw hiPSCs and maintain as undifferentiated cultures in mTeSR-based medium.
5. Prepare media using recipes below.

Note: Both the hiPSCs maintenance and differentiation media are good for up to 2 weeks at 4°C. Thus, it is not advisable to prepare all media at once, rather it is better to prepare it when needed at the specific step of the procedure.

⚠ **CRITICAL:** All procedures are performed in a BSL-2 certified laboratory biosafety cabinet with standard aseptic techniques. Cultures are grown and maintained in a humidified incubator at 37°C with 5% CO₂.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse anti-SOX2 (1:200)	Santa Cruz	Cat# sc-365823, RRID:AB_10842165
Mouse anti-OCT4 (1:200)	Santa Cruz	Cat# sc-5279, RRID:AB_628051

(Continued on next page)





Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Mouse anti-SSEA4 (1:200)	Abcam	Cat# ab16287, RRID:AB_778073
Rabbit anti-Nanog (1:100)	Abcam	Cat# ab21624, RRID:AB_446437
Mouse anti-NESTIN (1:500)	Santa Cruz	Cat# sc-23927, RRID:AB_627994
Rabbit anti-TUBB3, monoclonal (1:2000)	BioLegend	Cat# 801201, RRID:AB_2313773
Goat anti-CHAT, monoclonal (1:200)	Millipore	Cat# AB144P, RRID:AB_2079751
Mouse anti-HB9, monoclonal (1:500)	DSHB	Cat# 81.5C10-c
Rabbit anti-Islet1 (ISL1), monoclonal (1:250)	Abcam	Cat# ab109517; RRID: AB_10866454
Donkey anti-Mouse IgG (H + L), Alexa Fluor 488 (1:500)	Jackson ImmunoResearch	Cat# 715-545- 150, RRID:AB_2340846
Sheep anti-Mouse IgG (H + L), Alexa Fluor 594 (1:500)	Jackson ImmunoResearch	Cat# 515-515-062, RRID:AB_2340331
Donkey Anti-Rabbit IgG (H+L), Alexa Fluor 488 (1:500)	Jackson ImmunoResearch	Cat# 711-545-152, RRID:AB_2313584
Donkey Anti-Rabbit IgG (H+L), Alexa Fluor 594 (1:500)	Jackson ImmunoResearch	Cat# 711-585- 152, RRID:AB_2340621
Recombinant DNA		
pMDLg/pRRE	(Dull et al., 1998)	RRID: Addgene_12251
pRSV-Rev	(Dull et al., 1998)	RRID: Addgene_12253
pMD2.G	(Dull et al., 1998)	RRID: Addgene_12259
pCSC-NGN2-IRES-GFP-T2A-Sox11	(Liu et al., 2016)	RRID: Addgene_90214
pCSC-ISL1-T2A-LHX3	(Liu et al., 2016)	RRID: Addgene_90215
pCSC-Ngn2-IRES-ISL1-T2A-LHX3	(Sepehrmanesh and Ding, 2020)	N/A
Chemicals, peptides, and recombinant proteins		
mTeSR1 complete kit (basal medium plus 5× supplement)	STEMCELL Technologies	Cat#85850
Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM-F12)	Hyclone	Cat#SH30023.02
Dulbecco's Modified Eagle Medium (DMEM)	Gibco	Cat# 11965092
Neurobasal Media	Thermo Fisher Scientific	Cat#21103049
MEM Non-Essential Amino Acids Solution (NEAA) (100×)	Thermo Fisher Scientific	Cat#11140050
Glutamax (100×)	Thermo Fisher Scientific	Cat#35050
N2 supplement (100×)	Thermo Fisher Scientific	Cat#17502-048
B27 supplement (50×)	Thermo Fisher Scientific	Cat#17504-044
Retinoic acid (RA)	MilliporeSigma	Cat#R2625
Valproic acid (VPA)	MilliporeSigma	Cat#99-66-1
2-Mercaptoethanol (BME)	Thermo Fisher Scientific	Cat#21985023
Heat Stable Recombinant Human Basic Fibroblast Growth Factor (bFGF)	Gibco	Cat#PHG0367
Human EGF Recombinant Protein	Gibco	Cat# PHG0311L
Heparin	Thermo Fisher Scientific	Cat#BP252450
bFGF	PeproTech	Cat#100-18B
BDNF (Brain Derived Neurotrophic Factor)	PeproTech	Cat#450-02
GDNF (Glial Derived Neurotrophic Factor)	PeproTech	Cat#450-10
NT3 (Neurotrophic factor 3)	PeproTech	Cat# 450-03
Forskolin (FSK)	MilliporeSigma	Cat# F6886
DMSO, Dimethyl Sulfoxide	Thermo Fisher Scientific	Cat# D1391
KnockOut™ Serum Replacement	Thermo Fisher Scientific	Cat#10828010
Penicillin/streptomycin (Pen-Strep)	Thermo Fisher Scientific	Cat#15140122
Dulbecco (d) PBS (without calcium, magnesium)	Thermo Fisher Scientific	Cat#14190250
Triton X-100	MilliporeSigma	Cat#X100
Accutase (100 mL)	Corning	Cat#25-058-CI
Polyethylenimine (PEI)	Polysciences	Cat# 23966-2
Versene	Thermo Fisher Scientific	Cat#A4239101
Matrigel Matrix	Corning	Cat#356234
Bovine serum albumin (BSA)	Thermo Fisher Scientific	Cat# bp9706
Rho kinase (ROCK) inhibitor (Y-27632), 10 mM	MilliporeSigma	Cat#129830-38-2
TRIzol™ Reagent	Invitrogen™	Cat#15596026
SuperScript™ III First-Strand Synthesis System	Invitrogen™	Cat#18080051
Antifade Mounting Medium	Vector Laboratories	Cat#H-1400-10
PowerTrack™ SYBR Green Master Mix	Thermo Fisher Scientific	Cat#A46012

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
Postnatal day 1 (P1) pups	Charles River	C57BL/6(B6) Mouse Inbred 027
Experimental models: Cell lines		
Mice Primary Astrocytes	N/A	N/A
iPSC lines	Coriell Institute for Medical Research	Coriell Cat# GM23476, RRID: CVCL_T841
HEK293T cells	ATCC	ATCC Cat# CRL-3216, RRID: CVCL_0063
Oligonucleotides		
See Primer List in Table 1.	(Akter et al., 2021)	See Primer List in Table 1.
Other		
6-well plate	Corning	Cat#490007-412
Petri Dishes with Clear Lid	Thermo Fisher Scientific	Cat#FB0875713
Armadillo PCR Plate, 96-well T	Thermo Fisher Scientific	Cat# AB2496
24-well cell culture plates	Thermo Fisher Scientific	Cat#142485
0.22-μm vacuum filter	MilliporeSigma	Cat# SLGP033RB
0.45-μm vacuum filter	MilliporeSigma	Cat# SLHV033RB
15 mL polystyrene Conical tube	Thermo Fisher Scientific	Cat# 14-959-53A
50 mL polystyrene Conical tube	Thermo Fisher Scientific	Cat# 14-432-22

MATERIALS AND EQUIPMENT

All equipment and materials need to be sterilized or autoclaved. Any reagents or liquids that cannot be autoclaved need filter (0.22 μm) sterilization. We recommend all materials to be prepared freshly and stored at 4°C for no longer than two weeks. Scaling up and down according to the amount of medium needed.

iPSC culture medium		
Reagent	Final concentration	Amount
mTeSR1 Basal media	N/A	400 mL
5× supplement	1×	100 mL
Pen/Strep (100×)	1×	5 mL
Total	N/A	505 mL

⚠ CRITICAL: Store media at 2°C–8°C for up to 2 weeks or –20°C for up to 6 months. Avoid warming the medium in the 37°C water bath. Before use, take the desired amount of medium and warm it in biosafety cabinet at room temperature.

iPSC induction media		
Reagent	Final concentration	Amount
iPSC culture medium	N/A	~50 mL
Retinoic acid (RA, 100 mM)	10 μM	5 μL
Valproic acid (VPA, 1 M)	0.5 mM	25 μL
Total	N/A	50 mL

⚠ CRITICAL: Always prepare freshly before use. Store media at 2°C–8°C for up to 2 weeks or –20°C for up to 6 months. Before use, take the desired amount of medium and warm it in biosafety cabinet at room temperature.

⚠ CRITICAL: RA is a light sensitive compound. Work under low-light conditions when working with RA. The diluted stock can be stored at 4°C in dark. The 100 mM stock in 100% ethanol can be stored at –80°C for up to 2 years.



⚠ **CRITICAL:** RA is a potent teratogen. Harmful if swallowed and causes irritation to skin. Dispose concentrated stock solutions responsibly according to the manufacturer's recommendations.

⚠ **CRITICAL:** Valproic Acid (VPA) is a synthetic derivative of propylpentanoic acid with anti-epileptic properties. It causes irritation to eye, skin, harmful if swallowed.

Resuspension growth/KOSR media		
Reagent	Final concentration	Amount
DMEM/F12	N/A	~38.5 mL
KOSR (100%)	20%	10 mL
Glutamax (100x)	1 x	0.5 mL
NEAA (100x)	1 x	0.5 mL
BME (1000x)	50 μ M	50 μ L
Pen/Strep (100x)	1 x	0.5 mL
Total	N/A	50 mL

⚠ **CRITICAL:** Always prepare freshly before use. Store complete medium at 2°C–8°C for up to 2 weeks.

Neurosphere (NSP) medium		
Reagent	Final concentration	Amount
DMEM/F12	N/A	~48 mL
N2 supplement (100x)	1 x	0.5 mL
Pen/Strep (100x)	1 x	0.5 mL
Glutamax (100x)	1 x	0.5 mL
NEAA (100x)	1 x	0.5 mL
BME (1000x)	50 μ M	50 μ L
Heparin (5 mg/mL)	8 μ g/mL	80 μ L
bFGF (200 μ g/mL)	10 ng/mL	2.5 μ L
EGF (100 μ g/mL)	10 ng/mL	5 μ L
Total	N/A	50 mL

⚠ **CRITICAL:** Always prepare freshly before use. Store complete medium at 2°C–8°C for up to 2 weeks.

Neuron progenitor cell (NPC) medium		
Reagent	Final concentration	Amount
DMEM/F12 (1:1)	N/A	24 mL
Neurobasal	N/A	24 mL
Pen/Strep (100x)	1 x	0.5 mL
N2 supplement (100x)	1 x	0.5 mL
B27 supplement (50x)	1 x	1 mL
Glutamax (100x)	1 x	0.5 mL
NEAA (100x)	1 x	0.5 mL
BME (1000x)	50 μ M	50 μ L
bFGF (200 μ g/mL)	10 ng/mL	2.5 μ L
EGF (100 μ g/mL)	10 ng/mL	5 μ L
ROCK inhibitor (Y-27632, 10 mM)	10 μ M	50 μ L
Total	N/A	50 mL



⚠ **CRITICAL:** Always prepare freshly before use. Store complete medium at 2°C–8°C for up to 2 weeks.

NPC freezing medium		
Reagent	Final concentration	Amount
Complete NPC medium	N/A	30 mL
KOSR (100%)	30%	15 mL
DMSO (100%)	10%	5 mL
ROCK inhibitor (Y-27632, 10 mM)	10 μ M	50 μ L
Total	N/A	50 mL

⚠ **CRITICAL:** Always prepare freshly before use. Store complete medium at 2°C–8°C for up to 2 weeks.

Neuron maturation medium		
Reagent	Final concentration	Amount
DMEM: F12 (1:1)	N/A	24 mL
Neurobasal media	N/A	24 mL
Pen/Strep (100 $\times$)	1%	0.5 mL
N2 (100 $\times$)	1 $\times$	0.5 mL
B27 (50 $\times$)	1 $\times$	1 mL
FSK (20 mM)	5 mM	12.5 μ L
BDNF (20 μ g/mL)	10 ng/mL	25 μ L
GDNF (20 μ g/mL)	10 ng/mL	25 μ L
NT3 (20 μ g/mL)	10 ng/mL	25 μ L
Total	N/A	50 mL

⚠ **CRITICAL:** Prepare all stock aliquots of FSK, BDNF, GDNF, and NT3 at indicated concentration and keep them in –80°C freezer. Store the complete medium at 4°C up to 2 weeks.

Note: Medium compositions of NPC medium, Neurosphere medium, and maturation media were adapted from (Aker et al., 2021; Ding et al., 2021; Sepehrmanesh and Ding, 2020).

Astrocyte cell culture medium		
Reagent	Final concentration	Amount
Dulbecco's Modified Eagle Medium (DMEM)	N/A	~ 42.5 mL
Fetal bovine serum (FBS)	15%	7.5 mL
Pen/Strep (100 $\times$)	1 $\times$	500 μ L
Total	N/A	50 mL

⚠ **CRITICAL:** Always prepare freshly before use. Store complete medium at 2°C–8°C for up to 2 weeks.

HEK Cell culture medium		
Reagent	Final concentration	Amount
DMEM	N/A	~45 mL
FBS	10%	5 mL
Pen/Strep (100 $\times$)	1 $\times$	500 μ L
Total	N/A	50 mL

Store complete medium at 2°C–8°C for up to 2 weeks.



PEI solution stock

Reagent	Final concentration	Amount
PEI (transfection grade, 25 kDa, Linear)	1 µg/µL	50 mg
Milli-Q water	N/A	50 mL
Total	N/A	~50 mL

▲ **CRITICAL:** Dissolve PEI in sterile Milli-Q water. Incubate the tube at 55°C in a water bath and vortex until PEI is completely dissolved. Adjust pH to 7 if necessary. Cool PEI solution to room temperature (~25°C) and filter sterilized using a 0.22-µm vacuum filter. Aliquot and store at –80°C.

RT-PCR reaction condition

RT-PCR master mix

Reagent	Amount
PowerTrack™ SYBR™ Green Master Mix	6.25 µL
Forward primers	0.25 µL
Reverse primers	0.25 µL
cDNA	1 µL
Nuclease-free water	4.25 µL
Total RT PCR volume	12.00 µL

RT-PCR cycling conditions

Steps	Temperature	Duration	Cycles
Enzyme activation	95°C	2 min	1
Denature	95°C	15 s	40
Anneal/extend	60°C	60 s	
Melting curve analysis	Refer to instrument documentation		

STEP-BY-STEP METHOD DETAILS

Part 1: hiPSCs culture and expansion

⌚ Timing: 6–10 days

This part permits the expansion of hiPSCs for cryostorage and produces healthy pluripotent colonies suitable for neural differentiation. hiPSCs are cultured under feeder-free conditions and passaged when cells approach about 70% confluency.

1. Prepare Matrigel Matrix coated plates before starting the hiPSC culture. One 6-well plate (at 80% confluency, about 1 million cells per well) is sufficient to start the differentiation.
 - a. Thaw Matrigel Matrix at 4°C. Make smaller aliquots using refrigerated pipette tips. Aliquots can be stored at –80°C for long term storage.

Note: Matrigel is sensitive to temperature. When aliquoting Matrigel, place Matrigel and microcentrifuge tubes on ice within the biosafety cabinet. We suggest diluting Matrigel in DMEM-F12 (1:1) to prevent gelling during long term storage.

- b. Dilute Matrigel at 1:100 in cold DMEM-F12 medium. For example, add 100 µL Matrigel in 10 mL DMEM-F12 media.

Table 1. Primer list

Marker	Target	Forward/Reverse primer sequence (5'-3')
Pluripotency marker	SOX 2	AGGATAAGTACACGCTGCC/TTTCATGTGCGCGTAACTGTC
Pluripotency marker	NANOG	TGCTTCTGCTGAGATGCCT/CAGAAGTGGTTGTTGCCT
Pluripotency marker	KLF4	TCTCAATTGCTGACCCAT/CGGATCGGATAGGTGAAGCT
Differentiation marker	PAX6	GGGCGGAGTTATGATACCTACA/ATATCAGGTTCACTCCGGGAA
Differentiation marker	OTX1	TACGCCCTCCTTCTCTACT/GCATGTGGGTGGTGATGATG
Differentiation marker	DCN	CTGAAGAACCCTCACGCATTGA/GGCAATTCCTCAGCTGATTCT
Differentiation marker	IGF2	CAATATGACACCTGGAAGCAGT/GTAGAGCAATCAGGGGACGG
Differentiation marker	GATA2	ACCTGTTGTCAAATTGTGAGA/ATCCCTTCCTTCTCATGGTCA
Differentiation marker	SOX7	ACTCCACTCCAACCTCCAAG/TTTCATTGCGATCCATGTCCC
Differentiation marker	SOX17	ATCGGGGACATGAAGGTGA/TCCTTAGCCACACCATGAA
House-Keeping Gene	GAPDH	CAAATTCATGGCACCCTCA/GGACTCCACGACGTACTCAG

Note: If hiPSCs colonies show enhanced differentiation and low adherence, higher concentration of Matrigel (1:50) could be used for coating plates to facilitate cell attachment.

- c. Add 1 mL diluted Matrigel matrix to each well of the 6-well plate. Make sure to let Matrigel cover the entire surface of the well and incubate the plate at 37°C for minimum of 1 h.
- d. Aspirate Matrigel just before seeding cells.

Note: Avoid air dry of Matrigel-coated wells before seeding cells.

2. Rapidly thaw a hiPSC cryovial by gently swirling the vial in a 37°C water bath until there is just a small bit of ice left in the vial. In a laminar flow hood, transfer cells to a 15 mL conical tube containing 5 mL of pre-warmed iPSC culture medium mTesR1 containing 10 μ M ROCK inhibitor.
3. Spin cells at 200 $\times$ g for 3 min.
4. Remove supernatant and resuspend the cell suspension in mTesR1 medium containing 10 μ M ROCK inhibitor and transfer 2 mL of the cell suspension into each well of the Matrigel-coated 6-well plate.

Note: Resuspend cell pellet in a proper volume of culture medium depending on the cell numbers in the frozen stock. We recommend seeding cells ranging from 1×10^4 to 2×10^4 cells/cm².

5. Change medium every day. Cells will be ready for the first passage in 2–4 days according to the growth of the hiPSC line.

Note: At this point, the healthy hiPSC colonies will appear as tight colonies with smooth edges and are separated from each other (Figure 1A).

6. Once cells reach about 70% confluency, start the first cell passage by preparing another Matrigel-coated 6-well plate. This 6-well plate could be directly used for the iPSC induction.
7. Cells are passaged at 1:3 ratio. This way, two wells of the first 6-well plate (starting culture plate) can be passaged into a freshly prepared Matrigel-coated 6-well plate for iPSC induction.
 - a. Remove medium and wash wells with DPBS once.
 - b. Add 1 mL Versene to each well and incubate at 37°C for 6–7 min. The optimized incubation time varies from different hiPSC lines. For example, some hiPSC lines require 5 min while the genetically modified or patient derived hiPSC lines requires variable minutes.
 - c. Aspirate Versene from the wells. Add 2 mL mTesR1 medium to the wells and gently pipette up and down to dislodge the cells. Large aggregates of stem cell colonies should be seen floating in the medium. Gently triturate 2–3 times with a 5 mL pipette to break colonies into smaller pieces.

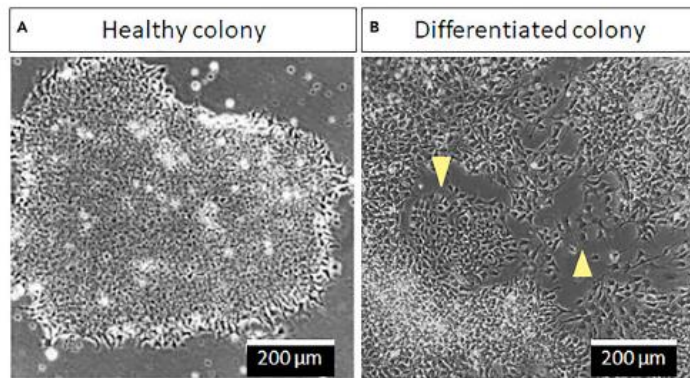


Figure 1. Images of healthy and differentiated hiPSC colonies

(A) Typical morphology of a healthy hiPSC colony with smooth edges.

(B) Differentiated hiPSC colonies usually appear as clusters of scattered and flat cells (arrowheads). Scale bars, 200 μ m.

Note: Versene is an EDTA solution to be used as a gentle non-enzymatic cell dissociation reagent and hence can be used for clump passaging. Typically, the cells remain attached at the bottom of the culture plate before adding neutralized medium.

⚠ CRITICAL: Avoid generating a single-cell suspension because it will result in differentiation and poor cell survival.

- d. Collect medium containing the cell colonies in a 15 mL tube and add extra mTesR1 medium up to 12 mL.
 - e. Gently mix by pipetting up and down 2 or 3 times. Transfer 2 mL cell suspension to each well of the newly Matrigel-coated 6-well plate.
 - f. Place 6-well plate in incubator and change iPSC culture medium daily.
8. The newly passaged 6-well plate should reach 80%–90% confluency in 3–5 days and can be used to start the iPSC induction.

Note: We recommend using hiPSCs culture within 2–4 passages from a frozen stock for this differentiation protocol.

⚠ CRITICAL: Make sure there are no differentiated colonies in these expanding cultures. These colonies show the loss of boundary intactness and can be identified by the apparent scattered morphology of cells (arrowheads in Figure 1B). If the differentiated cells only occupy a small fraction (<10%), remove them by scratching with a sterile pipette tip or careful aspiration with pipette tip attached to vacuum. If substantial differentiated cells are noticed, repeat the hiPSCs culture.

Part 2: iPSC induction

⌚ **Timing:** 7 days

The next step is to direct hiPSCs toward a neuroectodermal fate, which can be assessed by the generation of NESTIN⁺/PAX6⁺ neural progenitors.

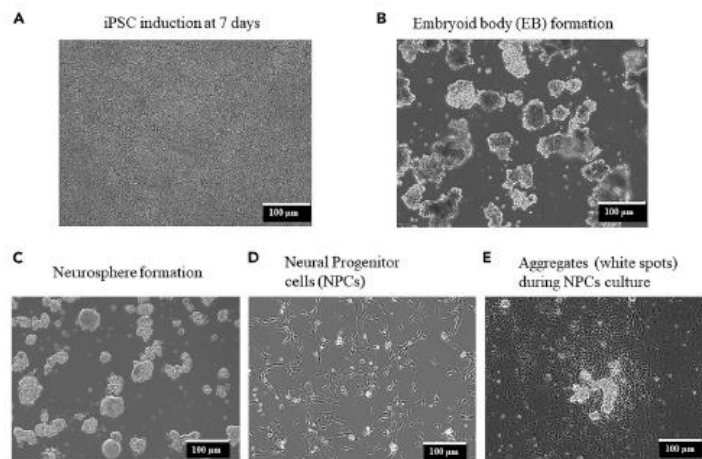


Figure 2. Micrographs of cells at different stages during the induction of hiPSCs to neural progenitor cells (NPCs)
(A) Typical morphology of hiPSCs cultured in induction medium at 7 days.
(B) Embryoid body (EB) formed in resuspension growth medium.
(C) Neurosphere (NSP) in NSP medium.
(D) Neural Progenitor cells cultured in NPC medium.
(E) Some aggregates (white dot) could be noticed in NPC cultures. Scale bars, 100 µm.

9. Prepare iPSC induction medium containing 10 µM RA and 0.5 mM VPA. Warm up to room temperature before use.
10. Aspirate hiPSCs culture medium from the 6-well plate and add 2 mL of iPSC induction medium to each well.
11. Every day, replace the medium completely with fresh medium for seven days.

Note: As cells are growing over time, more medium can be used to maintain the pH value (e.g., 3 mL per well of 6-well plate).

12. Cells should appear as a monolayer and grow together tightly at the end of day 7 (Figure 2A). High & tight confluency is optimal for resuspension growth in the next step.

Part 3: Embryoid body (EB) formation

⌚ Timing: 4 days

This stage requires ultra-low attachment 10 cm Petri Dishes which prevent cells from attaching to the bottom.

Note: Ultra-low attachment plates have a specialized surface coated with a hydrophobic hydrogel which inhibits cell attachment and forces them to remain in the suspension.

13. Prepare resuspension growth medium/KOSR medium with 10 µM Rock inhibitor.

Note: ROCK inhibitor Y-27632 has been shown to significantly inhibit apoptosis and enhance cell survival (Pettinato et al., 2015). The ROCK inhibitor could be added throughout the process for cell lines with poor growth.

14. Warm up resuspension growth medium/KOSR medium at room temperature.
15. Remove iPSC induction medium and wash wells once with DPBS.
16. Add 1 mL Versene to each well and incubate at 37°C for 6–7 min.
17. Remove Versene and add 1 mL of KOSR medium (resuspension medium) to each well. Gently shake the plates to dissociate cells. Scrape cells by using a 5 mL pipette if necessary.
18. Transfer cell suspension to a 15 mL tube.

Note: Triturate the cell suspension 1–2 times using a pipette to break apart the larger cell clusters into smaller aggregates.

19. Add 1 mL KOSR medium to rinse the well and pool together to the 15 mL tube.

Note: For 3 wells of 6-well plate, the total cell suspension volume is ~6 mL.

20. Add ~9 mL of KOSR medium to a low attachment Petridish. Transfer 6 mL cell suspension to the Petridish and gently mix.

Note: Check under microscope and most cells should be in aggregates. To let aggregates fully suspended, the total volume should be ~15 mL in each Petridish.

21. Change resuspension growth medium/KOSR medium every 2 days and culture cell aggregates for 4 days to form embryoid body (EB) (Figure 2B).
22. Change medium during suspension growth.
 - a. Gently swirl the plate and transfer cell suspension into a 15 mL conical tube (if more than 15 mL, split into two tubes).
 - b. Allow the cell aggregates to settle to the bottom of the tube (it will take about 10 min).
 - c. Remove supernatant (leaving ~1 mL) and add ~14 mL of fresh KOSR medium.
 - d. Carefully transfer cell suspension to the Ultra-low attachment Petridish.
 - e. Gently shake the petri dish to aid uniform distribution of the aggregates and place it back to the incubator.

Part 4: Neurosphere (NSP) formation

⌚ Timing: 7 days

23. Prepare Neurosphere (NSP) medium and warm it up at room temperature.
24. Transfer cell suspension (embryoid body at 4 days in step 22) to a 15 mL tube (if more than 15 mL, split into two tubes) and let aggregates settle down to the bottom of the tube.
25. Remove supernatant (leaving ~1 mL liquid) and add ~14 mL NSP Medium.
26. Carefully transfer cell suspension to the Ultra-low attachment Petridish.
27. Replace with fresh NSP medium every two days as describe in step 22 (~15 mL NSP medium/ 10 cm Petridish).
28. Cells are cultured in NSP Medium for 7 days to form neurospheres (Figure 2C).

Part 5: Generation of neural progenitor cells (NPCs)

⌚ Timing: 4–7 days

In this stage, neurospheres will be digested into singular cells and expanded to generate NPCs.

29. Prepare Matrigel-coated 10 cm culture plates as described in step 1.
30. Transfer NSP aggregates to a 15 mL tube and let aggregates sit down to the bottom (~10 min).



31. Remove supernatant (leaving ~1 mL liquid) and add 5 mL of DPBS to wash the aggregates.
32. Let aggregates sit down to the bottom (~10 min). Remove the supernatant and add 8 mL Accutase to NSP aggregates.
33. Incubate at 37°C for 15 min. Gently pipetting 5 strokes with a 5 mL pipette in the middle (at ~7 min) to break the large aggregates.
34. Add ~3 mL NPC medium to neutralize the digestion.
35. Gently pipette up and down several times to break most of aggregates into singular cells.
36. Spin cells at 200×g for 3 min.
37. Remove supernatant and resuspend cell pellet into appropriate volume of NPC medium.
38. Seed NPCs onto Matrigel coated 10 cm culture plates (Figure 2D).

Note: Neurospheres from one plate can give rise to 2–4 million cells and can be seeded onto four 10 cm plates.

39. Mix well and culture cells in incubator.
40. Change NPC medium every two days until cells are close to full confluency and then prepare NPC frozen stocks in the following stage.

Preparation of NPC frozen stocks

⌚ Timing: 1–2 h

When NPCs reach ~90% confluency, prepare for frozen stocks. At this stage, some small aggregates could be noticed in the culture plate. Remove these aggregates (white spots) by a filter pipette tip (Figure 2E).

41. Gently shake the medium and remove the supernatant. Wash once with DPBS.
42. Add 3 mL Accutase to each 10 cm plate and incubate at 37°C for 2–3 min. Gently shake the plate and most cells should be dissociated from the plate.
43. Add 3 mL NPC medium with rock inhibitor to neutralize the digestion. Pipette gently to dissociate cells from the plate.
44. Transfer cell suspension to a 15 mL tube. Spin down at 200×g for 3 min.

Note: To estimate the yield, an aliquot (~20 µL) could be taken from cell suspension for cell counting by a hemocytometer.

45. Remove supernatant and resuspend cell pellets into desired volume of precold (4°C) NPC freeing medium and aliquot quickly into cryovials.
46. Transfer cryovials into a freezing container and place it into a –80°C freezer. On the next day, transfer cryovials to a cryogenic storage container with liquid nitrogen for long-term storage.

Note: It is recommended to freeze NPC at a concentration of 1×10^6 /mL/vial. The yield of one 10 cm plate is about 4×10^6 NPCs.

Part 6: Lentivirus preparation for motor neuron (MNs) generation

This part describes the preparation of lentiviruses expressing transcription factors, which are necessary and sufficient to induce NPCs to MNs. To ensure high induction efficiency, it is critical to prepare high-quality supercoiled plasmids and make lentiviruses with good titers. For detailed information about lentivirus preparation and the applications, please see our previous studies (Ding et al., 2013, 2016, 2018; Ding and Kilpatrick, 2013). In this protocol, we used three lentiviral vectors,

pCSC-NGN2-IRES-GFP-T2A-Sox11 (Plasmid 1), pCSC-ISL1-T2A-LHX3 (plasmid 2), and pCSC-
Ngn2-IRES-ISL1-T2A-LHX3 (plasmid 3) (see [key resources table](#)).

Culture and expansion of HEK293T cells

⌚ Timing: 3–4 days

47. Quickly thaw a frozen stock of HEK293T cells in a 37°C water bath.
 - a. Transfer the cells to a 15 mL tube with 5 mL DMEM medium and centrifuge at 200×g for 3 min.
 - b. Discard the supernatant and re-suspend the cells in 10 mL fresh HEK Cell Culture medium.
48. Seed HEK293T cells into a 10 cm tissue culture plate. Grow cells at 37°C with 5% CO₂ in a standard tissue culture incubator.

Note: It is recommended to use low passage number (<20) HEK cells in the preparation of lentiviruses.

49. Once cells reach about 90% confluency, split cells for expansion.
 - a. Aspirate media from culture plate and wash once with 1× PBS.
 - b. Incubate cells with 0.5 mL of 0.05% Trypsin (0.25% Trypsin diluted with 1× PBS) for 2–4 min until cells have been fully detached.
 - c. Add 3 mL of HEK cell culture medium to inactivate the dissociation reagent.
 - d. Triturate the cells to create single cell suspension, collect the cells into a 15 mL tube and centrifuge the cells at 200×g for 3 min.
 - e. Remove the supernatant and resuspend the cell pellet into proper volume of HEK culture medium. Split cells in 10 cm plates at the ratio of 1:3 to 1:5.

Transfection and lentivirus preparation

⌚ Timing: ~4 days

The day before transfection, the HEK293T cells must be seeded at a proper cell density. Lentiviral plasmid DNA is delivered by the Polyethylenimine (PEI)-mediated transfection method.

50. Day 0: Seed about 8×10^6 HEK293T cells into each 10 cm culture plate for lentivirus production. Next day, HEK cells should be about 80% confluency for transfection.

⚠ CRITICAL: It is recommended to perform transfection (Day 1) in the late afternoon and replace the media in the following morning (Day 2). The longer exposure (>16 h) to transfection mixture may influence cell viability.

51. Day 1 (transfection):
 - a. Add the lentiviral plasmid and the packaging vectors to DMEM (no serum) as described in the recipe below. Scale up based on how many plates to be transfected.
 - b. Mix well by pipetting up and down for a few times.
 - c. Add desired amount of PEI into plasmid mixture and immediately mix thoroughly by pipetting up and down for 10 times or rigorously vortex for 15 s.
 - d. Incubate the mixture at room temperature (~25°C) for 15 min.
 - e. Add the mixture (1 mL/ 10 cm plate) dropwise to each plate with gentle rocking.
 - f. Gently mix by shaking up and down, and then left and right for a few times before putting the plate back into incubator.

Recipe of lentiviral transfection mixture (for one 10 cm plate)

Reagent	Amount	Volume
DMEM	N/A	1 mL
pMDLg/pRRE (1 µg/µL)	2 µg	2 µL
pMD2.G (1 µg/µL)	1 µg	1 µL
pRSV-Rev (1 µg/µL)	0.5 µg	0.5 µL
Desired plasmid (1 µg/µL)	4 µg	4 µL
Polyethylenimine (PEI)	1 µg/µL	22.5 µL

Note: The ratio of PEI to the total amount of DNA is 1:3. For example, the total amount of DNA is 7.5 µg for a 10 cm plate, add 22.5 µg (22.5 µL of 1 µg/µL) PEI into the plasmid mixture in 1 mL DMEM.

52. Day 2: After overnight (~16 h) incubation, gently replace medium with 10 mL HEK cell culture medium for each plate.

Note: It is recommended to warm up HEK cell culture medium to room temperature and carefully replace the medium to avoid cell detachment.

53. Day 3: Collect viral supernatants at about 48 h post-transfection (first collection). Add back 10 mL fresh HEK cell culture medium for each plate and incubate. Store the viral supernatant at 4°C.
54. Day 4: Collect viral supernatants at about 72 h post-transfection (second collection). Discard the plates in a biohazard bag and trash it after autoclave.
55. Pool two collections of the same lentivirus together and filter the viral supernatants with 0.45 µm vacuum filters. Store lentivirus at 4°C till use (within two weeks), or freeze at -80°C for longer term storage.

Note: Transfection efficiency can be examined under a fluorescence microscope if the lentiviral vector contains GFP reporter (e.g., plasmid #1). At lentivirus collection, most HEK cells (>90%) should be GFP (+) (Figure 3B).

Lentivirus titration

⌚ Timing: 4 days

Before transduction, we recommend to titer the virus to verify the quality. The virus can be titrated based on GFP reporter (plasmid#1) (Figures 4A and 4B) or immunocytochemistry (ICC) of lentivirus expressed factors (plasmid#2 and #3) (Figures 4C–4F) as previously reported (Ding and Kilpatrick, 2013; Sepehrimanesh et al., 2021).

56. Seed HEK cells in a 24 well plate at a density of 8×10^4 cells/well/0.5 mL medium. Cells will reach ~70% confluency the next day at the time of titration.
57. Make a serial dilution (e.g., 1:10, 1:100 etc.) of viral supernatant with HEK culture medium.
58. Add 50 µL of undiluted and diluted lentivirus to desired wells. Set one well as a negative control without transduction.
59. Change the medium after 8 h or overnight (~16 h) exposure to lentivirus.
60. Check the percentage of positive cells after 48–72 h.

Note: For lentivirus expressing GFP reporter, examine GFP (+) cells directly under a fluorescence microscope in living cells. For lentivirus that does not express fluorescent reporters, cells need to be fixed and perform ICC to examine lentivirus-expressed factors.

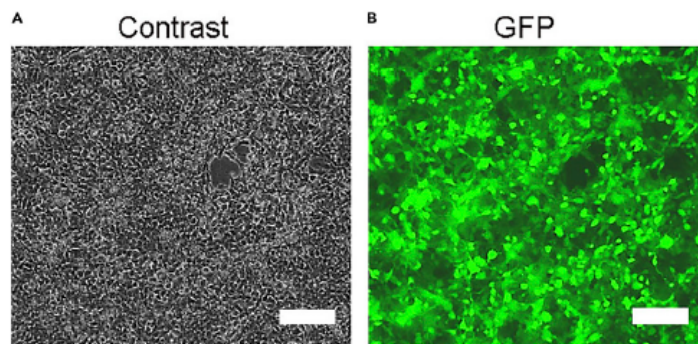


Figure 3. Transfection efficiency of plasmid with GFP reporter

(A) Micrograph of HEK cells (phase contrast) at virus collection.

(B) Micrograph of HEK cells (GFP) at virus collection (72 h post-transfection). Almost all HEK cells are transfected positive (plasmid #1 with co-expressed GFP). Scale bars, 100 μ m.

61. Transduced positive cells should be more than 90% in undiluted wells and more than 30% in 10-fold diluted wells (Figure 4).

⚠ CRITICAL: A good titer of lentiviral supernatants should be no less than 1×10^6 IFU/mL. (IFU, Infectious Unit).

Part 7: Lentiviral transduction of NPCs to generate MNs

⌚ Timing: 2–4 weeks

This part directs NPCs toward MNs by lentiviral delivery of transcription factors. This part can be done in two different ways. One approach is to deliver four transcription factors N-S-I-L (NEUROG2, SOX11, ISL1, and LHX3) with two lentiviruses. Another approach is to use a single lentivirus co-expressing three factors N-I-L (NEUROG2, ISL1, and LHX3). Both approaches can generate mature functional MNs. The second approach tends to generate MNs with higher purity and yield (Figure 6). These two approaches have been systematically examined and compared in our previous study (Sepehrmanesh and Ding, 2020).

62. Prepare Matrigel-coated 6-well culture plate as described in step 1.
63. Rapidly thaw NPC frozen cryovials by gently swirling the vial in a 37°C water bath until there is just a small bit of ice left in the vial. Transfer cells to a 15 mL conical tube containing 5 mL of pre-warmed NPC culture medium in a laminar flow cabinet.
64. Spin down cells at 200 $\times$ g for 3 min.
65. Gently resuspend cell pellets into proper volume of NPC medium and seed NPCs onto Matrigel-coated plates at a density of 3×10^4 cells/cm².

Note: One frozen vial with 1×10^6 NPCs is enough to seed 3 wells of a 6-well plate.

66. Next day, the NPC confluency is expected to be about 60%. Add about 300 μ L lentiviral supernatant to each well.

Note: At lentivirus transduction, the proper NPC density (50%–60% confluence) is critical to obtain higher yield of MNs. If two lentiviruses are used for transduction, add 300 μ L of each lentiviruses at MOI $\sim$ 2.

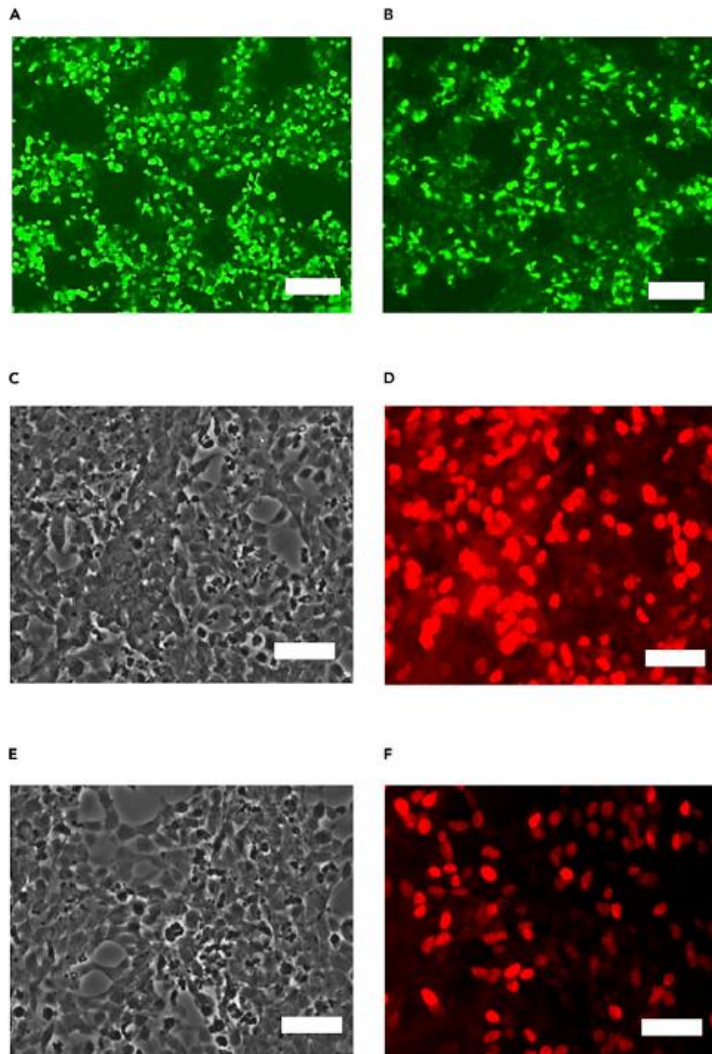


Figure 4. Lentivirus titration

(A) Micrograph of HEK cells transduced with undiluted virus expressing GFP reporter (plasmid #1) at 72 h post-transduction. Scale bar, 50 μ m.
 (B) Micrograph of HEK cells transduced with 1:10 diluted virus expressing GFP reporter (plasmid #1) at 72 h post-transduction. Scale bar, 50 μ m.
 (C) Micrograph of HEK cells (phase contrast) transduced undiluted virus (plasmid #3) and ICC with ISL1 antibody. Scale bar, 25 μ m.
 (D) Micrograph of HEK cells (fluorescent) transduced undiluted virus (plasmid #3) and ICC with ISL1 antibody. Scale bar, 25 μ m.
 (E) Micrograph of HEK cells (phase contrast) transduced 1:10 diluted virus (plasmid #3) and ICC with ISL1 antibody. Scale bar, 25 μ m.
 (F) Micrograph of HEK cells (fluorescent) transduced 1:10 diluted virus (plasmid #3) and ICC with ISL1 antibody. Scale bar, 25 μ m.



67. NPCs are exposed to lentiviruses for around 6 h to overnight (~16 h). Then replace the culture medium with Neuronal Maturation Medium.
68. Half change the medium every other day till analysis or replating.

Note: The age of iPSC-MNs is counted from the lentiviral transduction as dpi (days post-viral infection). Neuron-like morphology can be noticed after 3 dpi.

Preparation of coverslips with monolayer of astrocyte cells

⌚ Timing: 1–2 weeks

Mouse astrocytes were isolated from postnatal day 1 (P1) pups and cultured in DMEM with 15% FBS as reported (Schlodge et al., 2013). The Animal Procedure Statement in this study was approved by institutional animal care and use committee (IACUC) at University of Louisiana at Lafayette. The IACUC approval number is 2019-8717-027.

69. Preparation of astrocyte cells:
 - a. Prepare Matrigel-coated 10 cm culture plate as described in step 1.
 - b. Rapidly thaw frozen astrocytes in a 37°C water bath. Transfer cells to a 15 mL conical tube containing 5 mL of pre-warmed astrocytes culture medium in a laminar flow cabinet.
 - c. Centrifuge the cells at 200×g for 3 min.
 - d. Add 10 mL of astrocytes culture medium and gently resuspend cell pellet.
 - e. Seed astrocytes onto Matrigel-coated plates at a density of 3×10^4 cells/cm². One frozen vial with 1×10^6 astrocyte cells can seed a 10 cm plate.
70. Preparation of coverslips with a monolayer of astrocyte cells:
 - a. Prepare Matrigel-coated 24 wells plate with glass coverslips.
 - b. Once astrocyte cells reach about 90% confluency, aspirate media from astrocytes cell culture plate and wash once with 1× PBS.
 - c. Incubate cells with 3 mL 0.05% Trypsin in PBS for 2–4 min until cells have been fully detached.
 - d. Add 3 mL of astrocyte cell culture medium to inactivate the dissociation reagent.
 - e. Collect the cells into a 15 mL tube and centrifuge at 200×g for 3 min.
 - f. Remove the supernatant and resuspend cells in 12 mL of astrocyte cell culture medium.
 - g. Triturate the cells to create single cell suspension and seed onto Matrigel-coated coverslips (0.5 mL/well).

Replating MNs onto coverslips with monolayer astrocytes

⌚ Timing: 1–2 h

At 5 dpi, MNs can be replated and seeded onto coverslips with a mono-layer of astrocytes for long-term culture (more than 14 dpi) and further analysis.

71. Dissociate MNs from culture plates.
 - a. Aspirate media from the cell culture plate and wash once with 1× PBS.
 - b. For each well of 6 well plate, incubate cells with 1 mL of dissociation reagent (e.g., accutase) for 2–4 min until most of cells are detached.

Note: This step is critical for neuronal survival. Excess of accutase or longer incubation can be detrimental to neurons.

- c. Add 2 mL of DMEM: F12 medium to neutralize the dissociation reagent.
 - d. Collect the cells into a 15 mL tube and centrifuge at 200×g for 3 min.

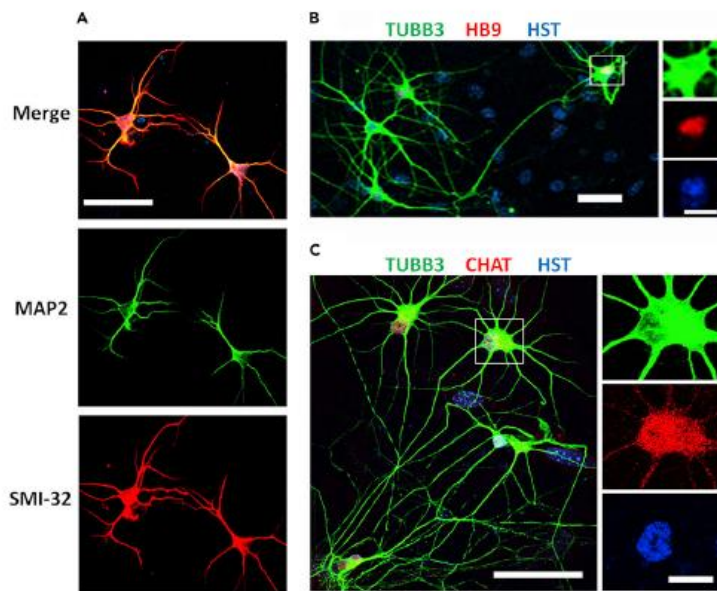


Figure 5. Verification of iPSC-MN identity

(A) Confocal micrographs of MNs at 7 dpi. MAP2 antibody recognizes dendrites and SMI-32 antibody recognizes neurofilaments. Scale bar, 50 μ m.
(B) Confocal micrograph of MNs at 10 dpi. Neuron marker TUBB3 shows the soma and neuron processes, HST (Hoechst 33342) stained nuclei, and nuclear HB9 was used as the early MN marker. The rectangle highlighted MN was also shown at a large magnification with separated channels. Scale bars, 50 μ m and inset 20 μ m.
(C) Confocal micrograph of MNs at 14 dpi. Neuronal marker TUBB3 shows the soma and neuron processes, HST stained nuclei, and choline acetyltransferase (ChAT) was used as a mature MN marker. The rectangle highlighted MN was also shown at a large magnification with separated channels. Scale bars, 50 μ m and inset 20 μ m. Adapted with permission from (Sepehrmanesh and Ding, 2020).

e. Remove the supernatant and resuspend cells gently in an appropriate volume of neuronal maturation medium.

72. Seed at a density of 1×10^3 cells/cm² dissociated MNs onto coverslips with astrocytes (0.5 mL/well).

73. Change half volume of Neuronal Maturation Medium (1:1) every other day till analysis.

Examination of MN identity

⌚ Timing: 2–3 days

MN identity can be verified by immunostaining of specific markers at different developmental stages. The survival, purity and yield can be calculated as our previous reports (Ding et al., 2021; Sepehrmanesh and Ding, 2020).

Neuronal morphology is examined under inverted microscopy directly in living cultures. Within 7 dpi, neurites will rapidly grow out from the soma displaying neuronal phenotype. At 14 dpi, coverslips are fixed and immunostained with neuron specific markers, such as TUBB3, MAP2, and neurofilament protein (SMI-32) (Figure 5).

To verify MN identity, immunostaining for HB9 and/or ChAT (Figure 5) are performed at different maturation stages. HB9 is an early MN marker and highly expressed at 10–20 dpi. ChAT is a mature MN marker gradually increase the expression at 14 dpi or later.

74. ICC MN markers:

- a. Take coverslips from 24-well plates at desired time points (7 dpi, 14 dpi, etc.)
- b. Fix cultured cells with 4% paraformaldehyde (PFA) in PBS (pH 7.4) and incubate at room temperature for 30 min.
- c. Wash twice with PBS.
- d. Incubate fixed cells in blocking buffer (PBS containing 0.2% Triton X-100 and 3% BSA) for 1 h for permeabilization and blocking.
- e. Incubate fixed cells with primary antibodies in blocking buffer (PBS containing 0.2% Triton X-100 and 3% BSA) at 4°C overnight (~16 h).

Note: The antibodies include anti-TUBB3 and anti-MAP2 as neuronal markers, anti-HB9 as an early MN marker, anti-CHAT as a late MN marker.

- f. After removal of primary antibodies, wash coverslips with PBS for 10 min × 3.
- g. Incubate fixed cells with corresponding fluorophore-conjugated secondary antibodies in dark, at room temperature for 2 h.
- h. Wash coverslips with PBS for 10 min × 3.
- i. Incubate cells with Hoechst 33342 (1 µg/mL in PBS) for 10 min to stain cell nuclei.
- j. Briefly wash coverslips with PBS twice.
- k. Mount coverslips with mounting medium on microslides.
- l. Air dry the coverslips at room temperature in dark (30 min to a few hours).

75. Visualize cells under the confocal microscope.

76. MNs purity could be calculated using the ratios of HB9 (+) cells to TUBB3 (+) cells, and/or CHAT (+) cells to TUBB3 (+) cells as previously reported (Ding et al., 2021; Sepehrmanesh and Ding, 2020).

EXPECTED OUTCOMES

Using this protocol, hiPSCs can be induced to NPCs (Figure 2), and then differentiated into MNs. To validate cells at different stages in culture, immunocytochemistry and/or quantitative RT-PCR analysis can be used to examine the expression of stage-specific genes. For example, hiPSCs highly express pluripotency markers such as OCT4, NANOG, SOX2 and SSEA4 (Akter et al., 2021). NPCs strongly express PAX6 and NESTIN (Liu et al., 2021). Successfully differentiated neurons robustly express generic neuron marker TUBB3, MAP2 and neurofilament protein (SMI-32) (Figure 5). The MN identity can be verified by the early MN marker nuclear HB9 (Figure 5A) and the late MN marker CHAT (Figure 5B). Compared with the use of two lentiviruses expressing four transcription factors (N-S-I-L), the optimized protocol using a single lentivirus expressing three factors (N-I-L) significantly improved the neuronal survival and the final iPSC-MN yield (Figure 6).

LIMITATIONS

The generation of iPSC-MNs from hiPSCs following this protocol is easy, reliable, and efficient. Preparation of high quality of hiPSCs and NPCs is prerequisite to successfully generate MNs. Transduction of NPCs with proper amount of lentivirus is critical to obtain MNs with high purity and yield. Using this protocol, we have successfully generated MNs using patient derived iPSC lines (Ding, 2022; Ding et al., 2021). However, the efficiency of this protocol may vary with different hiPSC lines and may require the optimization of transduction and culture conditions.

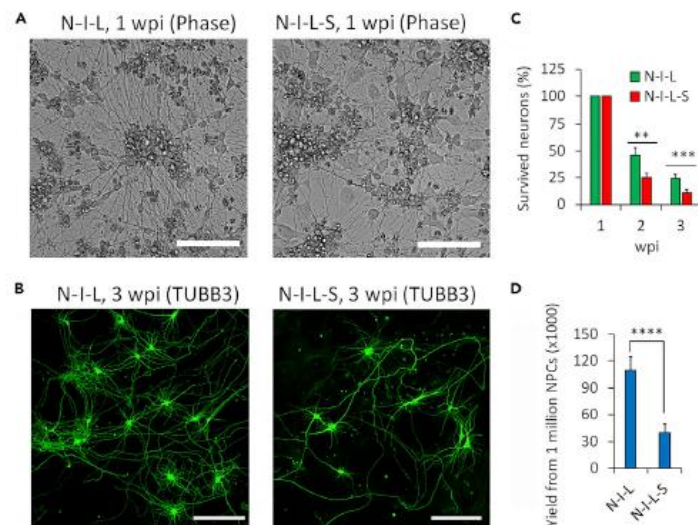


Figure 6. Optimized induction condition dramatically improved iPSC-MN survival and yield

(A) Phase contrast micrographs of iPSC-MNs induced via indicated combinations of transcription factors at 7 dpi. Scale bars, 100 μ m.

(B) Survived neurons under indicated conditions. The number of neurons at 1 wpi (weeks post-viral infection) was set as 100%. N, NGN2; S, mSox11; I, ISL1; L, LHX3. N (neurons) > 3,000 from triplicates. ** $p < 0.01$; *** $p < 0.001$.

(C) Representative micrographs of iPSC-MNs induced via indicated transcription factors at 3 wpi with immunostaining of TUBB3. Scale bars, 100 μ m.

(D) The yield of iPSC-MNs at 3 wpi from 1 million NPCs at transduction. N (biological replicates) = 3. **** $p < 0.0001$. Adapted with permission from (Sepehrimanesh and Ding, 2020).

TROUBLESHOOTING

Problem 1

Poor recovery of hiPSC frozen stocks (steps 1–4)

Poor recovery of hiPSC from frozen stocks can be manifested by more floating dead cells and very slow growth in the first several days after seeding.

Potential solution

Before starting the hiPSC culture, use freshly prepared Matrigel Matrix to coat plates at least 30 min at 37°C. Prewarm the culture medium to room temperature. Quickly thaw the frozen stocks in a 37°C water bath. Gently resuspend the cell pellets and transfer cell suspension to culture plates and minimize the physical damage of cells. These measures will improve the recovery of hiPSC from frozen stock.

Problem 2

Poor growth and more differentiation in hiPSC culture (steps 5–8)

Slow growth and substantial differentiated hiPSCs are observed.

Potential solution

To improve the growth and minimize the differentiation, use freshly prepared culture medium. Prewarm the culture medium to room temperature and change the medium daily.

Problem 3

Poor neural differentiation (steps 9–12)

Poor neural differentiation is observed when directing pluripotent hiPSCs toward a neuroectodermal fate.

Potential solution

In our hands, iPSC induction medium containing RA, and VPA is sufficient to direct hiPSCs toward a neuroectodermal fate. However, if poor neural differentiation is observed, SMAD inhibitors e.g., dorsomorphin (BMP signaling inhibitor, 1.5 μ M) and SB431542 (TGF beta inhibitor, 10 μ M) can be added to the iPSC induction medium. Inhibition of BMP/TGF β signaling has been shown to enhance neuroectodermal fate commitment in hESCs (Wattanapanitch et al., 2014).

Problem 4

Poor attachment during neuron progenitor cells generation (steps 29–40)

During NPC formation, the single-cell dissociation step can result in poor attachment and poor viability for certain cell lines, even with the addition of ROCK inhibitors. This issue will dramatically affect the efficiency and yield of NPCs and iPSC-MNs.

Potential solution

There are several factors need to be considered. For example, at the NSP stage, making single cells will lead to neuronal death and larger aggregates can lead to poor attachment. In such an instance, gentle pipetting can break down NSP aggregates into small aggregates and also avoid making single cells. Coating plates with appropriate concentration (1:100) of Matrigel is crucial for these steps.

Problem 5

Low titers of lentiviruses (steps 50–61)

Potential solution

This might occur due to multiple reasons. One reason could be poor growth of HEK293T cells. Hence, it is recommended to use low-passage cells and let the cells fully recover from frozen stock (passage at least twice) before setting transfection. Improper HEK cell density (too low or too high) also can result in low transfection efficiency. Secondly, low transfection efficiency of plasmids. It is advisable to check the quality of plasmids and transfection reagents. Proper ratio of helper plasmids, lentiviral vectors, and transfection reagent will improve lentivirus titers.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents can be directed to Dr. Baojin Ding (baojin.ding@suhs.edu).

Materials availability

The majority of materials used in this protocol are commercially available. The sequence of qPCR primers used for characterization of hiPSCs and NPCs were listed in Table 1.

Data and code availability

This study did not generate/analyze any datasets or code that were submitted to any other repositories.



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AUTHOR CONTRIBUTIONS

Conceptualization, B.D.; methodology, B.D. and M.A.; investigation, B.D., M.A., H.C., M.S., and M.A.H.; writing, B.D. and M.A.; funding acquisition and supervision, B.D.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Review

Modeling Movement Disorders via Generation of hiPSC-Derived Motor Neurons

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Abstract: Generation of motor neurons (MNs) from human-induced pluripotent stem cells (hiPSCs) overcomes the limited access to human brain tissues and provides an unprecedented approach for modeling MN-related diseases. In this review, we discuss the recent progression in understanding the regulatory mechanisms of MN differentiation and their applications in the generation of MNs from hiPSCs, with a particular focus on two approaches: induction by small molecules and induction by lentiviral delivery of transcription factors. At each induction stage, different culture media and supplements, typical growth conditions and cellular morphology, and specific markers for validation of cell identity and quality control are specifically discussed. Both approaches can generate functional MNs. Currently, the major challenges in modeling neurological diseases using iPSC-derived neurons are: obtaining neurons with high purity and yield; long-term neuron culture to reach full maturation; and how to culture neurons more physiologically to maximize relevance to in vivo conditions.

Keywords: hiPSC; motor neurons; small molecules; transcription factors; movement disorders



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1. Introduction

Movement disorders are a group of neurological conditions that cause either increased or decreased or slow movements. The movements may be voluntary or involuntary, and implicated in many neurological diseases, such as Dystonia, amyotrophic lateral sclerosis (ALS), Ataxia, Parkinson's disease [1], and so on. Motor neurons (MNs) are a remarkably powerful cell type in the central nervous system (CNS), and they are involved in both autonomic and voluntary movements. Most prior research of movement disorders was carried out using patient postmortem tissues or rodent models [2,3]. However, some subtle alterations in brain tissues and the dysregulations in specific neuronal subtypes may be transient and therefore difficult to preserve and detect in posthumous patient tissues [4]. While animal models provide insights into disease mechanisms, significant species-dependent differences exist, and animal models only mirror the limited aspects of the pathophysiology of human diseases. It is believed that these species-dependent differences caused the high failure rate in clinical trials that have been derived from successful results in animal models [5–7]. Additionally, when using posthumous patient tissues or rodent models, it is difficult to decipher the molecular pathogenesis via biochemical approaches, which require a large number of high-purity living neurons. Human induced pluripotent stem cells (hiPSCs)-derived neurons overcome the limited access to human brain tissues and provide an unprecedented approach to model human neurological diseases [8].

iPSC-based disease modeling starts from Yamanaka and his colleagues' groundbreaking studies, which demonstrated that somatic cells could be reprogrammed into pluripotent stem cells by ectopic expression of four transcription factors (Oct4 (O), Sox2 (S), Klf4 (K), and c-Myc (M)) under defined culture conditions [9,10]. An essential requirement for fulfilling the potential of hiPSCs is the ability to reliably differentiate into all three germ layers (ectoderm, mesoderm, and endoderm) and generate specific cell types with defined phenotypic traits [11–13]. The development of iPSCs offered a new approach for patient

specific stem cell-based research, bypassing the reliance on overexpression models, interspecies differences of animal models, and also ethical concerns of using human embryonic stem cells (hESCs) [14]. Given that iPSCs derived from somatic cells can preserve the donor's genetic background, less transplant rejection will occur when they are used for cell therapy. The *in vitro* phenotypes of disease-specific iPSC-derived cells hold the most promise to bridge the gap between the clinical phenotype and fundamental molecular and cellular mechanisms, creating new strategies for drug screening and novel therapeutic interventions [15,16]. Additionally, CRISPR engineering of iPSCs enables researchers to make paired patient mutation lines and isogenic control lines, greatly facilitating the research for understanding disease pathogenesis [12,13,17–19].

It is also well known that iPSCs and iPSC-derived cells have limitations, especially in the context of culture heterogeneity and dosage variability. The best route of administration and survivability in the hostile inflammatory microenvironment is controversial [20]. Improved ways of making cells, gene-editing technologies, along with patient-derived iPSC cells, have revolutionized the generation of experimental disease models. This provides an unlimited supply of any type of cells from once-inaccessible human tissues for research [21,22]. Neurodevelopmental and neurodegenerative disorders are particularly approachable using iPSC technology as iPSC-derived neurons retain the patient's genomic context and provide an excellent cellular model system in deciphering the pathophysiology of diseases. Modulating the combination, concentration, and exposure time of crucial signaling molecules has yielded protocols for generating neurons and glia from iPSCs [23]. Thus far, researchers have developed protocols by which several neural and glial cell types could be generated, including glutamatergic neurons [24], GABAergic neurons [25,26], dopaminergic neurons [27], serotonergic neurons [28], MN [29–32], astrocytes [33], and microglia [34,35]. The improvement techniques and updated protocols in neural differentiation and maturation greatly facilitate the generation of iPSC-derived cells.

On the other hand, iPSC-based disease modelling is relatively new in biomedical research. Substantial progress has been made in developing differentiation protocols for the generation of different cell types. Excitingly, some *in vitro* models can recapitulate aspects of neuronal synaptic networks, which greatly advances functional modeling of neurodevelopmental and neurodegenerative diseases. Recent studies generating iPSC-derived MNs from movement disorders of ALS [36–38] and DYT1 patients [8,31,39] showed great potential to experimentally model molecular events underlying disease pathogenesis. The generation of patient-specific MNs provides an unprecedented approach in modeling MN-related disorders and deciphering cellular and molecular pathogenesis. In this review, we focused on the techniques for the generation of hiPSC-derived cholinergic MNs.

2. Generation of hiPSC-Derived MNs

Many studies are attempting to generate mature MNs from hiPSCs for modeling movement disorders. Developmental biologists have identified the signaling molecules and transcription factors that are involved in MN differentiation and maturation, providing the foundation for the generation of hiPSC-MNs. Currently, scientists are using these extrinsic factors to guide the MN differentiation from stem cells, thereby mimicking regionalization processes during nervous system development. Many protocols have been developed that rely upon core signaling pathways, which could synchronize neuronal induction to MN-specific signaling cascades and upregulate the expression of MN-specific genes.

The generation of hiPSC-MNs is a consecutive process that consists of a variety of induction stages, including iPSC induction, embryoid body (EB) formation, neural rosette growth, neuron progenitor cells (NPCs) differentiation, and MN induction and maturation (Figure 1A). At each induction stage, different culture media with different supplements will be employed to support and drive the cell fate towards MNs. At each differentiation stage, the cells show different growth patterns with unique cellular morphology and express specific protein markers (Figure 1B). These features can be used to verify the cell identity at each induction stage and perform quality controls to obtain highly pure MNs. In

the following sections, we will discuss the process of iPSC-MN generation with a focus on the approaches using small molecules (chemicals) and lentiviral delivery of transcription factors. Meanwhile, the regulatory mechanisms of MN differentiation at different stages will be discussed.

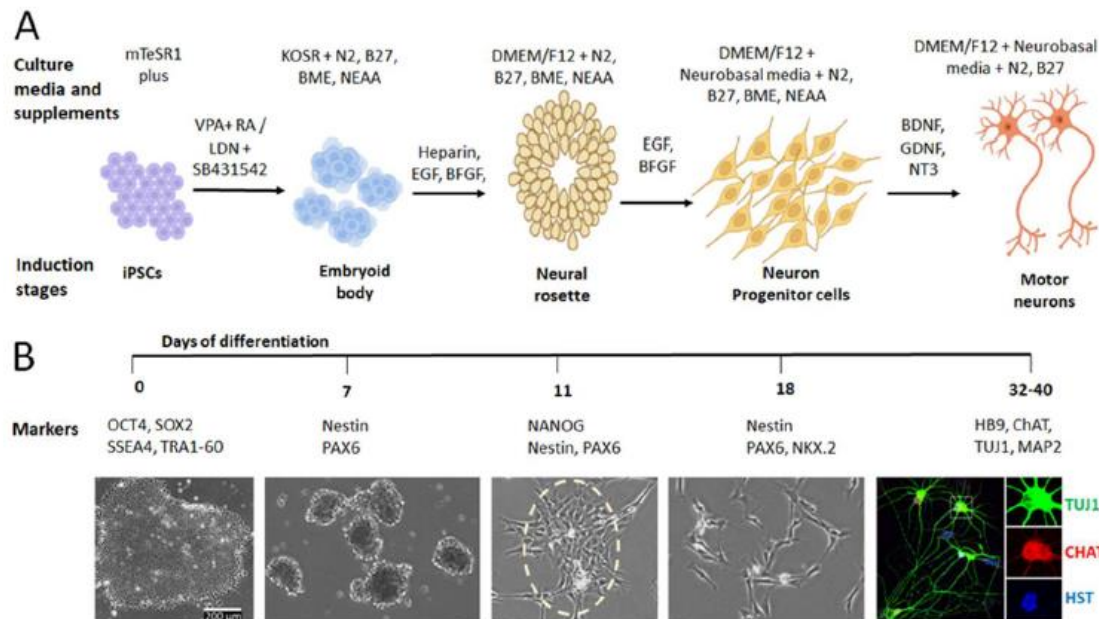


Figure 1. Generation of iPSC-derived MNs. (A) Schematic shows the process of generating iPSC-MNs. The culture media with different supplements are shown at different induction stages. (B) The timeline of the induction process from hiPSC to MNs. At each stage, specific markers and typical cellular morphology are shown. Image of MNs was adapted from [32].

2.1. Generation of NPCs from hiPSCs

Human nervous system development begins with neural induction converting ectodermal cells into neuroectodermal cells, leading to the formation of the neural plate and finally the neural tube [40,41]. How neural regionalization is precisely controlled to form the forebrain, midbrain, hindbrain, and spinal cord along with the rostral-caudal (R-C) axis remains unknown. Dorso-ventral, rostral, and caudal patterning are very important events in neural tube maturation and specification. The gradients of various morphogens such as the canonical WNT/ β -catenin signaling pathway (Wnt), retinoic acid (RA), and Sonic Hedgehog (Shh) signaling are involved in the determination of neuroaxis formation [42–44]. Insights from the model animals show that the rostral-caudal (R-C) axis patterning of NPCs is controlled by modulating the Wnt and RA signaling, whereas dorso-ventral patterning is controlled by the modulation of Shh signaling [45–48]. Most neuronal differentiation schemes mimic embryonic developmental signals by small molecule patterning. This initial study further showed that different combinations of small molecules used as patterning factors could push NPCs toward distinct neuronal fates [49–51]. The neuroectoderm is specified by inhibition of mesoderm and endoderm differentiation factors and acquires an initial rostral neural character through the regulation of BMP (bone morphogenetic protein), TGF β (transforming growth factor- β), FGF (fibroblast growth factor), and Wnt signaling [52–57]. The neural fate can be potentially induced by the inhibition of BMP and TGF β . These rostral neural progenitors are caudalized in response to Wnts, FGFs, and RA during early development [4].

One of the many ways of generating different types of neurons is through the differentiation of NPCs, which are a homogenous, multipotent, undifferentiated, self-renewable cell

population. NPCs are committed to become neural lineage and to be differentiated to specific neuronal types in defined culture conditions [58]. iPSCs are induced to become NPCs through neuralization, either by EB formation [59–62], or by dual SMAD inhibition in monolayers [49,63,64]. EB formation is the most widely used method for neuralization [65–69]. In this process, cells grow in suspension and spontaneously arrange in three-dimensional aggregates upon withdrawal of factors promoting pluripotency. The Dual-SMAD-Inhibition method was developed in 2009 by Chambers and colleagues [49]. This method used a small molecule (Noggin, SB431542) to suppress the TGF- β /Activin/Nodal pathway and the BMP-canonical pathway [70,71]. After a 5-day exposure of the hiPSCs to Noggin/SB431542, the cells became an early-stage neuroepithelial population with expression of SOX1, Paired box protein 6 (PAX6), and Zic family member 1 (ZIC1) markers, and were able to form neural rosette organization [49,72,73]. This protocol showed an 80% efficiency of hESC and hiPSC differentiation into PAX6-positive NPCs. Once the iPSCs cells differentiate into NPCs, the newly formed NPCs continue expressing neuroepithelial markers, such as SOX1 (SRY-Box Transcription Factor 1), SOX3 (SRY-Box Transcription Factor 3), (PSA-NCAM) Polysialylated-neural cell adhesion molecule, and MS1 (Musashi RNA Binding Protein 1) [74–76]. Reinhardt and colleagues reported a protocol that NPCs can be generated using only small molecules. Neural induction was introduced through inhibition of both BMP and TGF- β signaling using Dorsomorphin and SB43152. To stimulate the canonical Wnt signaling, CHIR99021, a GSK3 β inhibitor, was added to the cell medium, and the Shh pathway was stimulated by using purmorphamine. These neural progenitors are also able to differentiate into different types of neurons, including MNs [70].

Recently, two approaches were reported for generation of NPCs using a combination of small molecules: either RA and VPA (Valproic Acid) or LDN-193189 (SMAD inhibitor) and SB431542 (Activin/BMP/TGF-beta Pathway Inhibitor) [29,77,78]. Both combinations can successfully generate NPCs, which can be spontaneously differentiated into neurons consisting of glutamatergic (~75%), GABAergic (~15%), and dopaminergic (TH+) (~5%) neurons under defined culture conditions. These NPCs can also be differentiated into highly pure (90%) cholinergic MNs via transduction of three transcription factors: NEUROG2 (Neurogenin 2), ISL1 (LIM homeobox 1), and LHX3 (LIM homeobox protein 3) [29,31,32].

2.2. MN Induction via Small Molecules

Small molecules are bioactive compounds that can modulate specific cellular pathways involved in cell signaling, transcription, metabolism, or epigenetics, all of which are modulated during cellular reprogramming. If selective epigenetic modulation can be achieved with chemicals, it could remodel the chromatin structure and activate the gene expression of transcription factors, achieving similar effects to the ectopic expression of reprogramming factors. The advantage of using small molecules in reprogramming is that their biological effects are typically rapid, reversible, and dose-dependent, allowing precise control over specific outcomes by fine-tuning their concentrations and combinations. In Table 1, we summarized the roles of small molecules, including chemicals and peptide growth factors, in MN differentiation. These small molecules target different signaling pathways (Table 2) that promote MN differentiation and maturation. In Table 3, we summarized the chemical cocktails that have been reported in recent publications for the generation of iPSC-MNs.

Table 1. Roles of small molecules in neuron differentiation.

Chemicals	Functions	References
Retinoic acid (RA)	Agonist for RA receptors. Promotes neural differentiation.	[14]
Valproic acid (VPA)	Histone deacetylase inhibitor. Facilitates the reprogramming of fibroblasts into iPSCs. Promotes neuronal differentiation.	[79]

Table 1. Cont.

Chemicals	Functions	References
SB431542	Inhibitor of TGF- β , Activin and Nodal signaling. Differentiation of human ES and iPSCs into neural progenitors. Increase in reprogramming efficiency in combination with other small molecules.	[49]
CHIR99021	Selective inhibitor of glycogen synthase kinase 3 (GSK-3). Enables reprogramming of fibroblasts into iPSCs. Induces neuronal differentiation.	[49]
Purmorphamine (PUR)	Sonic Hedgehog (Shh) activator. Improves the efficiency of MN differentiation.	[80]
Dorsomorphin	Inhibitor of both activin/nodal/TGF- β and BMP pathways. Induces rapid and high-efficiency neural conversion in both hESCs and hiPSCs. Induces neuronal differentiation in vitro.	[81]
Y-27632	Highly potent and selective inhibitor of Rho-associated, coiled-coil-containing protein kinase (ROCK). Improves embryoid body (EB) formation efficiency. Enhances survival of hESC during cell passaging.	[82]
Forskolin (FSK)	Stimulates adenylate cyclase activity and increases cAMP. Regulates neuronal specification and promotes axonal regeneration.	[83]
Compound E	NOTCH signaling inhibitor. Accelerates MN maturation.	[84]
Brain-derived neurotrophic factor (BDNF)	Activates TrkB signaling. BDNF enhances the survival and differentiation of neurons in vitro. Critical for neuronal survival, morphogenesis, and plasticity.	[85]
Glial cell line-derived neurotrophic factor (GDNF)	Activates tyrosine kinase receptor signaling. Promotes neuronal differentiation in later culture periods. Potential roles in various pathways, mediating growth, differentiation, and migration of neurons.	[86]
Ciliary neurotrophic factor (CNTF)	Neurotrophic factor. Promotes the survival of different neurons and the differentiation of neural progenitor cells (NPCs) in vitro.	[87]
Neurotrophin-3 (NT3)	Neurotrophic factor-mediated Trk receptor signaling. Neurotrophic factors promote the survival of neurons. Growth factor involved in stem cell differentiation.	[88]
Basic fibroblast growth factor (bFGF)	Fibroblast growth factor family. Stimulates hESC to form neural rosettes. Supports the maintenance of undifferentiated human hESCs.	[89]
Epidermal growth factor (EGF)	Mitogen. Induces the in vitro and in vivo proliferation of neural stem cells, their migration, and their differentiation towards the neuroglial cell line.	[90]
Heparin	Promotes the growth of hESCs. Supports the binding of FGF to its receptor and increases the stability of FGF. Activates Wnt signaling for neuronal morphogenesis.	[91]

Table 2. Signaling pathways participating in MN differentiation.

Cell Signaling	Functions	References
Sonic Hedgehog (Shh) signaling	Shh signaling is required for the final specification of MNs. Activator: Purmorphamine (PUR); Inhibitors: Cyclopamine and HPI-1.	[92]

Table 2. Cont.

Cell Signaling	Functions	References
Dual SMAD inhibition	Block endodermal and mesodermal cell fates and promote neural conversion. Drive the rapid differentiation of hESCs and hiPSCs into a highly enriched population of NPCs. Inhibitors: SB431542, LDN193189, Noggin, LY364947, RepSox, Dorsomorphin, DMH-1	[93]
Neurotrophic factors signaling	Improves MN survival and maturation. Activators: BDNF, GDNF, NGF, NT-3	[94]
Wnt/ β -catenin signaling	Contributes to patterning, proliferation, and differentiation throughout vertebrate neural development. Activator: CHIR99021 (CHIR), Kenpaullone, SB216763 Inhibitor: APCDD1, Wai1/5T4	[95]
Notch signaling	Regulates the balance between MN differentiation and the maintenance of the progenitor state. Activator: Dll, Dll4 Inhibitor: DAPT, LY411575	[96]

Table 3. Chemical cocktails used for MN differentiation from iPSCs.

Chemical Cocktail and Cytokines	Target Signaling Pathways	Cellular Markers	Efficiency	Days	References
Chir-99021, SB431542, LDN193189, RA, SAG, DAPT, BME, Ascorbic Acid, and Y-27632	Wnt, FGF, SHH signaling	OLIG2, ISL1/2, HB9, LHX1/2, FOXF2	~77%	14	[68,97]
RA, PUR, Y-27632, VPA and CHIR99021	SHH signaling, WNT/ β -catenin	HB9, ISLET-1, ChAT	~85%	30–40	[98]
SB431542, CHIR99021, RA, PUR, BDNF, GDNF	SHH, WNT/ β -catenin, and Notch	TUJ1, MAP2, HB9, ChAT, SYP	>85%	28	[99]
SB 431542, CHIR99021, dorsomorphin, and Cpd E	Activin/nodal/TGF- β and BMP pathways, SHH signaling	ChAT, HB9, SOX11, PAX6, nestin, OLIG2, TUJ1, MAP2	~88%	21	[100]
SB 431542, dorsomorphin, BDNF, RA, and ISL1/2	TGF- β , Activin, Nodal, and canonical	FOXP1, OXA5, MAP2, TUJ1	>40%	24	[101]
SB 431542, CHIR99021, dorsomorphin, and RA	Activin/nodal/TGF- β , BMP and GSK-3	ChAT, HB9, SMI-32	~80%	24	[102]
SB 431542, dorsomorphin, B18R, synTFs mRNAs of neurogenin and NeuroD families, FSK, BDNF, GDNF, and NT-3	Activin/nodal/TGF- β and BMP pathways	ChAT, HB9, and ISL1	~86%	12	[103]
RA, SAG, BDNF, GDNF, and DAPT	Neurotrophic factors, canonical signaling	ChAT, HB9, ISL1, SMI-32, TUJ1	70–95%	32	[104]
Dorsomorphin, SB431542, CHIR99021, RA, PUR, ascorbic acid, dibutyryl cAMP	Activin/nodal/TGF- β and BMP pathways	OLIG2, SOX2, ISLET1, AP2, HB9, SMI32, TUJ1	~70	45	[105]
SB431542, DMH1, CHIR99021, RA, PUR, Cpd E	BMP, Activin, WNT, SHH and NOTCH	NKX2.2, OLIG2, ISL1, MNX, TUJ1, ChAT, BTX	>90%	28	[84]
PUR, RA	Shh, Agonist for retinoic acid receptors	HB-9, TUJ1, OLIG2	>85%	28	[106]
Compound C, RA, cAMP Neurotrophic factor	BMP and Activin signaling	TUJ1, MAP2 Synapsin I, HB9, ChAT	~70%	20	[107]
PUR, RA	Shh signaling agonist	HB9, ISL1/2, ChAT, OLIG2	~80%	15	[108]

To acquire the MN progenitor identity, NPCs need to caudalize and ventralize with the action of RA and Shh, respectively [42,109]. Shh induces the expression of homeodomain transcription factors and basic helix-loop-helix (bHLH) [11], the critical intermediaries in the

control of cell patterning and neuronal cell fate determination [110–113]. The combined actions of RA and Shh are thought to establish a spatial and temporal regulation of the expression of transcription factors, such as LHX1 (homeodomain transcription factors) [114–116], OLIGO2 (oligodendrocyte transcription factor), MNX1 (MN and pancreas homeobox 1, HB9), and ISL1 [42]. All of these factors are necessary for the subsequent differentiation of NPCs to MNs [117–119]. Several studies have identified that OLIG2 is a bHLH protein, which is essential for establishing MN progenitor identity downstream of Shh signaling. OLIG2 also has a key role in specifying the subtype identity and pan-neuronal properties of developing MNs [117,120–122]. The expression of downstream transcriptional regulators, particularly HB9, a homeodomain protein expressed in postmitotic MNs, is crucial to consolidate MN identity [123–125].

Small molecules have a profound influence on neural induction and promote hiPSC differentiation into MNs combined with the simultaneous inhibition of TGF β activin, Nodal, and BMP (also known as dual SMAD inhibition) signaling. Dual SMAD inhibition is a well-established method which utilizes small molecules to block endodermal and mesodermal cell fates and promote neuroectoderm conversion. It dramatically enriches neural ectoderm from pluripotent cells with a high expression of PAX6 and sex-determining region Y-box 2 (SOX2). Noggin and SB431542 are the most commonly used dual SMAD inhibitory small molecules. Noggin acts as a BMP inhibitor and SB431542 inhibits the Lefty/Activin/TGF β pathways by blocking the phosphorylation of ALK4, ALK5, and ALK7 receptors. The GSK-3 β inhibitor promotes neural progenitor proliferation by stimulating the canonical Wnt signaling pathway, which contributes to the maintenance of neural precursors. Molecular activation of SHH by Purmorphamine, canonical WNT signaling, and neural patterning by RA have been critical for MN induction. Previous studies have found that Shh induces upregulation of transcription factors OLIG2, NK2 homeobox 2 (NKX2.2), and neurogenin2 (NGN2) to direct the expression of MN fate-consolidating genes such as HB9 and ISL1. Neurotrophic and growth factors, such as BDNF, GDNF, and NT3 are also used as supplements to facilitate MN growth, maturation, and survival [8,29,126]. The entire differentiation process requires from 15 days to up to 2 months to generate fully functional MNs. In 2002, Wichterle and colleagues reported that RA and Shh were used to differentiate mouse ESCs into MNs through EB formation [127,128]. Wada and colleagues differentiated hESCs from the human and monkey ESCs toward MNs through neural rosette formation. They treated ESCs with 1 μ M RA and 500 ng/mL Shh, leading to neural precursors becoming Tubulin β III+, Hb9+, Islet1+, and choline acetyltransferase-positive (ChAT+) neurons [129].

Scientists have been working continuously to advance our understanding of MN differentiation and to improve the techniques for generating MNs from iPSCs. The rapid advancement of RNA sequencing technologies contributed to a deep understanding of transcriptome composition and has discovered a large number of non-coding RNAs (ncRNAs) that participate in MN differentiation (Table 4). These ncRNAs have intense regulatory activities in a wide range of biological processes, including neuronal development, subtype diversification, specification, differentiation, and function [130–132]. Among the ncRNAs, long non-coding RNAs (lncRNAs) and miRNAs (microRNAs) are especially abundant in the nervous system and have been shown to be implicated in MN development and function.

Table 4. Noncoding RNAs and their functions in MN differentiation.

Noncoding RNA	Function	References
miR-9	miR-9 modifies MN columns by a tuning regulation of transcription factor FoxP1 (Forkhead box protein 1) levels in developing spinal cords.	[133–135]
miR-218	Expression of miR-218 is directly upregulated by the Isl1-Lhx3 complex, which drives MN fate. Inhibition of miR-218 suppresses the generation of MNs in both chick neural tube and mouse embryonic stem cells.	[136,137]

Table 4. Cont.

Noncoding RNA	Function	References
mir-17~92	Confers MN subtype survival during development.	[136,138,139]
mir-27	mir-27 as a major regulator coordinates the temporal delay and spatial boundary of Hox protein expression, which contributes to the specification of MN subtype identity.	[140]
miR-183-5p	miR-183-5p is a central regulator of MN survival under stress conditions. Increased miR-183-5p is correlated with cell stress in MNs of ALS in pre-symptomatic and early symptomatic stages.	[141,142]
miR-196	The timing and rostro-caudal extent of Hoxb8 activity in the neural tube is tightly regulated by miR-196, a miRNA species encoded within three Hox gene clusters. miR-196 effectively suppresses endogenous Hoxb8.	[143]
miR-375	miR-375 facilitates human spinal MN development and protects MNs from DNA damage-induced degeneration.	[130,144,145]

Mature miRNAs are ~22-nucleotide single-stranded RNAs that can recognize the 3' untranslated region (UTR) of its target mRNAs and negatively regulate gene expression post-transcriptionally [146–148]. miRNAs are an integral part of the genetic program controlling MN survival and acquisition of subtype-specific properties [137,138,149]. Several studies also demonstrated that miRNAs mediated post-transcriptional regulation participates in fine-tuning the program of MN progenitor specification [148,150], MN differentiation [151–153], and subtype diversification [134–137,154,155]. miR-9 is involved in fine-tuning the differentiation of MN subtypes. Notably, a recent study revealed that miR-9 is transiently expressed during MN differentiation and regulates the expression of FoxP1 (Forkhead Box P1) and HOX (Homeobox (HOX) transcription factors. These transcription factors play a critical role in coordination of MN subtype identity and connectivity. In mice, overexpression of miR-9 induces neuronal differentiation by inhibiting the nuclear receptor [134,135], suggesting that miR-9 plays a role in fine-tuning the process of specification of MN subtype identity. Other studies showed that ISL1 expression by Olig2 transcription factors (OC1) was important to generate LMC MNs [133,156,157]. Studies showed that miR-9 and OC1 are in mutually exclusive patterns in the embryonic spinal cord and miR-9 efficiently represses OC1 expression, demonstrating that regulation of OC1 by miR-9 is a crucial step in the specification of spinal MNs.

miR-218 is the most abundant and highly enriched miRNA in developing and maturing MNs [136,137,158]. miR-218 is decreased in human ALS postmortem spinal cord, and cell-free miR-218 can serve as a marker for MN loss in a rodent model of ALS [159,160]. Studies showed that in the developing spinal cord, the expression of miR-218 is directly upregulated by the Isl1–Lhx3 complex, which drives MN fate. Inhibition of miR-218 suppresses the generation of MNs in both chick neural tube and mouse ESCs, suggesting that miR-218 plays a crucial role in MN differentiation [137]. Previously, it has been found that complete loss of miR-218 results in the breakdown of neuromuscular synaptogenesis, hyperexcitability, post-natal lethality, MN loss, and complete paralysis [136,154,155,161].

Chen and colleagues demonstrated that the repression of Olig2 mRNA (MN progenitor marker) is controlled by mir-17-3p microRNA [138,150]. The expression of miR-17-3p is repressed by Shh, which results in elevated expression of Olig2. Thus, a high amount of Shh will direct neuronal progenitors to differentiate toward MNs [162,163]. Functional studies indicate that miRNA plays a significant role in a broad range of cellular and developmental processes of subsets of MN. By using an in vitro model of human spinal MN development, it has been shown that miR-375 is strongly activated during spinal motor neurogenesis and its expression is specific to MNs [130]. Knockdown of miR-375 significantly impairs MN differentiation, highlighting its essential role in MN development. miR-375 also protects MNs from DNA damage-induced degeneration by inhibiting p53 and therefore preventing

apoptosis. Downregulation of the miR-375-3p in patients with spinal muscular atrophy leads to an increase of the p53 protein level and thus to apoptosis [144,145].

Long non-coding RNAs (lncRNAs) are RNAs that exceed 200 nucleotides in length, and they are not translated into proteins. lncRNAs participate in various stages during MNs differentiation, including guiding neural fate choice by driving transcription factor localization [164–166], regulating local translation at synapses [167,168], influencing MN development, and contributing to the pathogenic mechanisms underlying MN diseases (MNDs) [164,169].

The lncRNA CAT7 (chromatin-associated transcript 7) is a polyadenylated lncRNA that lies upstream (~400 kb) of MNX1 (MN homobox1). CAT7 has been found to temporally regulate MNX1 expression during the early stages of human ESC-MN differentiation. Loss of CAT7 causes de-repression of MNX1 before committing to motor neuronal lineage [170]. Another lncRNA Meg3 plays a critical role in maintaining postmitotic MN cell fate by repressing progenitor genes that regulate the differentiation of MN identity [171–173].

NEAT1 (nuclear-enriched abundant transcript 1) is a well-characterized lncRNA that functions as a chromatin regulator and organizes nuclear structures called ‘paraspeckles’. Paraspeckles contain proteins involved in transcription and RNA processing [174,175]. NEAT1 is highly enriched in neurons of the anterior horn of the spinal cord and in the cortical tissues of ALS patients. Nishimoto and colleagues demonstrated NEAT1 upregulation and increased paraspeckle formation in the MNs during the early phases of ALS pathogenesis [176–178]. The exact role of NEAT1 still needs to be resolved. Given their dynamic expression patterns in MNs and emerging roles in MN development and function, it is not surprising that dysregulation of noncoding RNAs has been implicated in MNDs. Understanding the mechanisms of action and functions of lncRNAs may assist the development of new therapies for MNDs.

2.3. MN Induction via Lentiviral Delivery of Transcription Factors

Previous reports have underscored the essential roles of transcription factors in MN development (Table 5). Decades of developmental studies have identified key signaling molecules and cell-intrinsic transcriptional programs [179,180] that specify MN identity during embryonic development. These transcription factors were transduced into neural progenitor cells after differentiation from ESCs/iPSCs, and MNs can be obtained 11 days after the transduction. These transcription factors are generally introduced into the cells via viral transduction (generally the most efficient ones) [29,181]. This transcription factors-mediated differentiation has been shown to produce highly efficient functional MNs with repetitively firing MNs after two weeks post-viral infection (wpi) [31,32].

Table 5. Roles of transcription factors in MN differentiation.

Transcription Factor	Functions	References
Neurogenin 2 (NEUROG2)	Transcriptional regulator and actively involved in neuronal differentiation. Unique and critical role in determining MN cell-type identity.	[182]
Sex determining region Y-box 2 (SOX2)	Critical for early embryogenesis and for maintaining embryonic stem cell pluripotency.	[183]
ISL LIM homeobox 1 (ISL1)	ISL1 is a major transcription factor necessary for MN identity. Fusion protein Isl1–Lhx3 specifies MN fate differentiation.	[184]
LIM homeobox 3 (LHX3)	Transcriptional activator involved in the development of interneurons and MNs.	[184]

Table 5. Cont.

Transcription Factor	Functions	References
POU class 5 homeobox 1 (POU5F1)	Critical for early embryogenesis and for embryonic stem cell pluripotency. Master regulator of initiation, maintenance, and differentiation of pluripotent cells.	[80]
Achaete-scute family basic helix-loop-helix transcription factor 1 (ASCL1)	Promotes cell cycle exit and develops neuronal progenitors and differentiation when expressed in neural progenitor cells.	[185]
POU Class 3 Homeobox 2 (POU3F2)	Plays potential role in morphological complexity, maturity, and action potentials of the neuronal cells	[186,187]

Transcription factors ISL1 and LHX3 are sufficient to activate a MN gene expression program in other neural progenitors and increase post-mitotic specification by directly reprogramming pluripotent stem cells into MNs [188–190]. Specifically, a combinatorial expression of LIM homeodomain transcription factors Lhx3 and Isl-1, together with the expression of the pro-neural gene NGN2, have been shown to be critical to induce MN specification during development [114,191–193]. The combinatorial expression of Lhx3 and Isl1 will form an Isl1-Lhx3-hexamer, which will trigger MN specification in a chick spinal cord, ESCs, and iPSCs [114,194–197]. The binding of the Isl1-Lhx3 complex activates the transcription of genes that are essential for MN specification such as HB9 and promotes the expression of a wide range of terminal differentiation genes, including a battery of cholinergic pathway genes that enable cholinergic neurotransmission [198,199].

The combination of different transcription factors was often used to obtain a high quality of iPSC-MNs. These transcription factors need to be delivered into cells via lentiviral vectors or other vehicles (Table 6). In 2013, the group of Hynek Wichterle demonstrated that overexpression of three transcription factors (Ngn2, Isl1 and Lhx3) was sufficient to rapidly and efficiently program spinal MN identity from the mouse ESCs. Replacement of Lhx3 by Phox2a (Paired Like Homeobox 2A) led to the specification of cranial, rather than spinal MNs. Isl1-Lhx3 and Isl1-Phox2a heterodimers showed different DNA-sequence preferences for the basis of cell reprogramming, indicating that there are synergistic interactions between programming factors underlying specification of alternate MN fates [189,200–202]. Goto et al. have used a single sendai virus-mediated overexpression of the TF cocktail NGN2, ISL1 and LHX3 in both mice and human iPSCs to promote the expression of MN markers. Notably, after 3 weeks of differentiation, NGN2/ISL1/LHX3-overexpressing neurons were electrophysiologically active and formed neuromuscular junctions (NMJ) with cultured myocytes [203]. The MNs derived via this method from ALS patient's iPSCs have also shown disease phenotypes. De Santis et al. expressed transcription factors of Ngn2, Isl1, and Phox2a in human iPSCs via Piggy-bac transposable vectors and converted human iPSCs into cranial MNs and upregulated pan-MN genes such as TUBB3, ISL1, and ChAT within the first 3 days of differentiation. HB9 expression was increased when LHX3 was co-expressed, whereas PHOX2B and TBX20 (T-Box Transcription Factor 20) were detected by day 5 [144]. Finally, the authors of this study functionally characterized the cranial MNs obtained after 12 to 13 days to observe that these cells were capable of firing action potentials upon current stimulation, and almost half of all analyzed cells even displayed spontaneous glutamatergic postsynaptic currents.

Table 6. Transcription factors used in generation of iPSC-MNs.

Transcription Factors Delivered	Delivery Vector	Cellular Markers	Efficiency	Days to Reach Maturation	References
NGN2, ISL1, LHX3	Lentiviral	MAP2, SMI32, TUBB3, HB9, ChAT	>95%	35 days	[29,32]

Table 6. Cont.

Transcription Factors Delivered	Delivery Vector	Cellular Markers	Efficiency	Days to Reach Maturation	References
NGN2	Lentiviral	ChAT, HB9, SMI-32, ISL1, FOXP1, MAP2, TUJ1	~95%	30 days	[204]
NGN2, SOX1, ISL1, and LHX3	Lentiviral	HB9, ChAT, TUBB3, MAP2, and synapsin	45–50%	35 days	[29,31]
POU5F1(OCT4) and LHX3	Lentiviral	MAP2, TUJ1, HB9, ChAT	70 ~ 90%	28 days	[205]
NGN2, ISL1, LHX3 and NGN2, ISL1, PHOX2A	Piggy-bac transposable	PHOX2B, TUJ1, ISL1, ChAT	~90%	11–12 days	[144,206]
NGN2, ISL1, LHX3	Sendai virus	HB9, MAP2, ChAT, TuJ1,	~93%	14 days	[203]
NGN2, ISL1, LHX3	Adenoviral	HB9, CHAT, SMI-31, HOXC6	60–70%	30 days	[191]
Ascl1, Brn2 (POU3F2), Myt1l, Hb9 (MNX1), NGN2, ISL1, and LHX3	Retroviral	MAP2, vChT, HB9, ISL1	~60%	35 days	[187]

Goparaju et al. showed that overexpression of NGN1 (Neurogenin 1), NGN2 (Neurogenin 2), NGN3 (Neurogenin 3), NEUROD1 (Neuronal Differentiation 1), and NEUROD2 (Neuronal Differentiation) in human PSCs combined with RA, forskolin, and dual SMAD inhibition via SB431542 and dorsomorphin yields highly pure neuronal cultures expressing the MN markers HB9, ISL1, and ChAT [103]. MNs generated from this method have shown functional activity (repetitive action potentials and calcium-transient) within a week. We have reported that a single lentiviral vector expressing three factors (NGN2, ISL1, and LHX3) is necessary and sufficient to induce iPSC-derived MNs (iPSC-MNs) [29,32]. MNs derived using these methods robustly expressed general neuron markers, such as microtubule-associated protein 2 (MAP2), neurofilament protein (SMI-32), tubulin β -3 class III (TUBB3), and MN-specific markers HB9 and CHAT. These MNs showed electrical maturation within 3 weeks [32].

3. Quality Control: Validation of Neuron Identity and Purity

To ensure that the high quality and purity of MNs can be obtained from hiPSCs, it is necessary to validate the cell identity at each stage during the process of induction and differentiation. Specific markers at different stages could be examined and used to estimate the induction quality and the MN purity (Figure 1B). As the passage number of iPSC may affect the differentiation of iPSC-derived neurons [207], using a lower passage number is recommended in the generation of hiPSC-derived MNs.

3.1. Markers of Early Induction from hiPSC to NPC

Patient-specific somatic cells, such as skin fibroblast cells and peripheral blood mononuclear cells (PBMC), can be reprogrammed into the pluripotent state through ectopic expression of Yamanaka factors (Oct3/4, Sox2, Klf4, and c-Myc). The iPSC must be fully characterized to ensure quality before differentiation into neurons or other cell types. The morphology of iPSCs should demonstrate a typical hESC-like appearance composed of tightly packed cells in phase contrast microscopy (Figure 1B). A healthy iPSC line should robustly express the pluripotency markers (SSEA4, TRA-1-81, OCT3/4, and SOX2) and show a normal karyotype [12,13]. There are several techniques that can be used to assess the pluripotency. The pluripotency markers could be examined at protein levels by immunocytochemistry (ICC) and western blot or by using real-time PCR to measure transcription levels. Newly generated iPSC lines need to be validated that they can differentiate into three germ layers: endodermal, mesodermal, and ectodermal cell lineages. This can be verified by two approaches: 1) in vitro differentiation of iPSCs in suspension to form EBs, which highly express trilineage markers [59,208], and 2) EBs injected into immunocompromised NOD/SCID mice will form teratomas, which consist of three germ layers [209,210]. Similarly, three germ layers specific markers can be examined using ICC or western blot at protein level or RT-PCR at transcription level. The iPSCs and differentiated cells need to be confirmed as mycoplasma-negative before establishing a cell line stock.

3.2. Markers of MNs at Early Immature Stages

To achieve more accurate disease modeling and maximize the potential applications, quality controls are critical to verify cell identity and purity. Many methods could be used, including specific marker expression, molecular and functional properties, cellular morphology assay, electrophysiological analysis, and animal transplantation. Many protocols analyze samples within 2 to 5 weeks from the onset of differentiation. Early stages of MNs showed the typical polygonal cell body with few and short dendrites (Weeks 1–2). However, the generation of ISL1/2 and HB9-expressing cells can vary from 3 days to 2 weeks after differentiation. Longer differentiation protocols have an advantage over shorter ones when preparing fully functional mature neurons, which could be used to examine the electrophysiological activity and synaptic network. After treatment with lentivirus-expressing-specific transcription factors or small molecules, usually within a week the cell will become neuron-like with condensed nuclei, long axons, and multiple neurites. Generation of MNs requires the identification of genes that are expressed at the initial stages of MN differentiation. At day 13 of differentiation, studies observed the expression of early MN-specific factors, PAX6, OLIG2, ISL-1 and NEUROD [191]. PAX6 and OLIG2 are required to initiate a general MN fate differentiation [43,117,119]. Studies have also found that ISL1 is the earliest marker involved in the establishment of MN fate. The expression of MN-specific genes becomes evident at week 3 or later, including strong expression of LHX3, ISL1, and HB9. Early MNs are commonly characterized by transient co-expression of HB9 and ISL1/2 [123,211]. Homeobox gene HB9 is required for the consolidation of MN identity, and its expression is restricted to somatic MNs of the hypoglossal nucleus. Transcriptional upregulation of MN markers such as ISL1, HB9, and OLIG2 was shown in the early stage of MN differentiation in several studies.

3.3. Markers of MNs at Late Mature Stages

As HB9 is downregulated over the course of MN maturation, the expression of choline acetyltransferase (ChAT) appeared and temporally increased [212]. As ChAT is an enzyme responsible for the synthesis of neurotransmitter acetylcholine in cholinergic MNs; the expression of ChAT indicates that the cells reach maturation stage. miR-218, abundantly and selectively expressed in maturing MNs, is being recently used as a molecular marker to identify MNs [136,137].

The final maturation can be achieved with the action of neurotrophic factors (BDNF, GDNF, NT3) and demonstrated by increased dendrites arborization and cell-cell connections (weeks 2–6). Mature MNs display larger soma, increased cell shape, complexity of neurite outgrowth, and electrophysiological properties. iPSC-derived MNs are generally considered mature after 3 weeks of differentiation [100]. Higher expression of neuronal markers of TUBB3, MAP2, non-phosphorylated neurofilament heavy chain (SMI32), ChAT, and vesicular acetylcholine transporter (vAChT) indicates the maturation of the MNs. Monitoring the electrophysiological status of MNs in vitro is currently the most comprehensive method to assess their maturation. To demonstrate maturation, MNs are Synapsin-positive and electrophysiologically active. The electrophysiologically mature MNs are able to fire repetitive action potentials and generate spontaneous activity that requires the development of intrinsic (e.g., sufficiently polarized resting membrane potentials) and extrinsic (e.g., synapse formation) properties. Mature MNs are also capable of recreating NMJs when cocultured with myotubes in vitro and expressing acetylcholine receptor (AChR) clusters. All these characteristics indicate that hiPSCs had efficiently differentiated into fully functional MNs.

4. Modeling Neurological Diseases Using hiPSC-Derived MNs

Modeling MN-related diseases using hiPSC-based approaches requires culture conditions in a dish that can recapitulate the events underlying MN differentiation, maturation, aging, and degeneration. Several protocols exist to generate MNs from hiPSCs, and these cells have been used to study the pathophysiology of MN-related diseases, such as ALS, spinal muscular atrophy, and DYT1 dystonia [213–218]. The known disease-dependent cell-

lular deficits are excellent features that can be used to validate the hiPSC-MNs in modeling the disease. For example, using patient-specific MNs, we have identified disease-dependent cellular deficits in DYT1 dystonia, including abnormal nuclear envelope morphology, disrupted neurodevelopment, impaired nucleocytoplasmic transport, and mislocalized nuclear Lamin B1 [31,39]. For each preparation of DYT1 iPSC-MNs, we routinely examined these cellular features to ensure that the materials we used are valid in modeling DYT1 dystonia. To identify and characterize functional molecular features of MNs, different technologies have been employed, ranging from fluorescence-based antibody staining of target markers to RNA-seq analysis. We usually perform ICC, Western blot, PCR, and qRT-PCR to verify the expression levels of neural markers at different stages during the induction process. Examination of well-characterized molecular markers is useful for determining the accuracy and efficiency of a protocol. Whole-transcriptome sequencing or RNA-seq, single cell sequencing is also often used to further characterize the expression of these markers in an unbiased manner. Modeling ALS with iPSC-derived MNs has recapitulated several known pathological findings in patient-derived cells, reinforcing the high value of the approach. Compared with healthy controls, patient iPSC-derived cells can be used to identify disease phenotypes at different levels, including molecular profiles, cellular features, and physiological functions. The development of high-throughput single-cell transcriptomics has changed the paradigm, empowering rapid isolation and profiling of MN nuclei, revealing remarkable transcriptional diversity within the skeletal and autonomic nervous systems. In addition, patient-specific iPSCs may also serve as powerful resources for personalized medicine, including drug discovery, genetic testing, and ultimately cell replacement therapy.

5. Future Challenges and Perspectives

Generation of hiPSC-MNs overcomes the limited access to human brain tissues and provides an unprecedented approach for modeling MN-related diseases, offering an excellent platform for developing therapeutic treatments. However, several challenges arise from using this cellular system for disease modeling [32]. First, the purity and yield of iPSC-derived neurons. Current human iPSC-MN induction protocols vary in timescale (ranging from 15 days to more than six weeks) and efficiency, with few protocols achieving both high efficiency and rapid MN generation [219,220]. Some studies require a large number of MNs with high purity, such as transcriptomic studies, to elucidate the alterations of genome-wide gene expression and proteomic studies to identify dysregulated factors in diseased neurons. Second, to obtain fully functional and mature neurons. Because of the lack of simplified and consistent protocols, the generated hiPSC-derived MNs in most studies are often functionally immature and heterogeneous. Some disease-dependent cellular and molecular deficits cannot be noticed until neurons reach full maturation, especially for age-related neurodegenerative diseases such as ALS, Alzheimer's disease (AD), and Parkinson's disease (PD). Modelling these late-onset diseases usually requires the long-term culture of neurons from several weeks to a few months, during which the neuron survival and potential contaminations are huge challenges. Third, how to culture the neurons more physiologically? Although chemical or physical modifications of the cell culture plates, such as coating with extracellular matrix (ECM), have been shown to be an efficient method to better mimic *in vivo* cell behavior [221], the outcomes of some experiments using *in vitro* cellular systems could be very different from the studies using *in vivo* models. iPSC-derived neurons cocultured with glial cells and the development of hiPSC to brain organoids under three-dimensional culture conditions could maximize the relevance to *in vivo* conditions.

To resolve these issues, studies are needed to understand the precise regulatory mechanisms of neural differentiation and maturation using *in vivo* models. Meanwhile, the protocols for the generation of iPSC-derived neurons need to be updated, simplified, and finally standardized to obtain consistent outcomes for biomedical research. The generation of different neuronal subtypes requires different protocols consisting of different

induction factors, specific culture conditions and supplements, and particular treatments and processes. Generally, induction efficiency and neuron survival are paramount to achieve good yield and purity. The combinations of small molecules and/or transcription factors will enhance the induction efficiency and maximize the purity, while the optimized culture conditions will promote neuron maturation and survival, which will lead to a higher yield. One major difficulty in obtaining fully mature iPSC-MNs is the long-term culture issue, in which both neuron survival and potential contaminations are huge challenges. We generate iPSC-MNs using lentiviral delivery transcription factors, and these MNs can reach full maturation at 3 wpi with characterization of high expression of presynaptic proteins, cholinergic markers, and firing action potentials. For setting up experiments that require fully mature neurons, we usually culture iPSC-MN for 4 wpi [29]. For such a long-term culture, culture media supplemented with neurotrophic factors and neurons cocultured with astrocytes are required. For chemical-induced iPSC-MNs, once the MN identity is verified, in theory, the neurons will reach full maturation after culture for a long enough time. However, we have not directly compared the maturation, the culture time, and the detailed characterizations of iPSC-MNs that are generated by different approaches. It is hard to say which approach is better than the other. Excitingly, the techniques of genome-editing and three-dimensional culture of brain organoids greatly expanded the applications of hiPSC in disease modeling, cell therapy, and drug development [222,223].

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Abbreviations

ALS	Amyotrophic lateral sclerosis
ASCL1	Achaetes-scute family bHLH transcription factor 1
ATRA	All-trans retinoic acid
bHLH	Basic helix-loop-helix
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
BDNF	Brain-derived neurotrophic factor
CNS	Central nervous system
Wnt	Canonical WNT/ β -catenin signaling pathway
CNTF	Ciliary neurotrophic factor
ChAT	Choline acetyltransferase
Cpd E	Compound E
CAT7	Chromatin associated transcript 7
EGF	Epidermal growth factor
ESCs	Embryonic stem cells
FGF	Fibroblast growth factor
DLB	Dementia with Lewy bodies

FOXP1	Forkhead box protein 1
FGF2	Fibroblast growth factor 2
GDNF	Glial cell line-derived neurotrophic factor
GSK-3	Glycogen synthase kinase 3
hiPSCs	Human induced pluripotent stem cells
HOXC6	Homeobox C6
HB9	Homeodomain
HST	Hoechst 33342
ISL1	Insulin gene enhancer 1
LHX3	LIM/homeobox 3
LHX1	Lim homeodomain transcription factors
LncRNAs	long non-coding RNAs
miRNAs	microRNAs
MAP2	Microtubule Associated Protein 2
MNs	Motor neurons
MS1	Musashi RNA Binding Protein
MYT1L	Myelin transcription factor 1 like
MXN1	Motor neuron and pancreas homeobox 1
NPCs	Neural progenitor cells
NGN2	Neurogenin-2
NMJs	Neuromuscular junctions
DAPT	N-[N- (3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester
NKX2.2	NK2 homeobox 2
NT3	Neurotrophin-3
ncRNAs	Non-coding RNAs
NEAT1	Nuclear-enriched abundant transcript 1
NGN1	Neurogenin 1
NGN2	Neurogenin 2
NGN3	Neurogenin 3
NEUROD1	Neuronal Differentiation 1
NEUROD2	Neuronal Differentiation 2
Olig2	Oligodendrocyte transcription factor
OC1	Onecut transcription factors
PSA-NCAM	Polysialylated-neural cell adhesion molecule
POU5F1	POU class 5 homeobox 1
PHOX2A	Paired like homeobox 2A
POU3F2	POU class 3 homeobox 2
Pax6	Paired box protein 6
PUR	Purmorphamine
qPCR	Quantitative PCR
RA	Retinoic acid signaling pathway
ROCK	Rho-associated coiled-coil containing protein kinase
Shh	Sonic Hedgehog signaling pathway
Sox2	Sex determining region Y-box 2
SAG	Smoothed agonist
SOX1	SRY-Box Transcription Factor 1
SOX3	SRY-Box Transcription Factor
SOX11	SRY-box transcription factor 11
Synapsins	Regulation of neurotransmitter release at synapses
TGFβ	Transforming growth factor-β
TUBB3	Tubulin Beta 3 Class III
TBX20	T-Box Transcription Factor 20
UTR	3'Untranslated region
VPA	Valproic Acid
VACHT	Vesicular acetylcholine transporter
WPI	Weeks post viral infection
DLB	Dementia with Lewy bodies

ALS	Amyotrophic lateral sclerosis
APCDD1	Adenomatosis polyposis downregulated
1ASCL1	Achaetes/scute family bHLH transcription factor 1
ATRA	All-trans retinoic acid/bHLH, Basic helix-loop-helix
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
BME	β -mercaptoethanol
BDNF	Brain-derived neurotrophic factor
CNS	Central nervous system
Wnt	Canonical WNT/ β -catenin signaling pathway
CNTF	Ciliary neurotrophic factor
ChAT	Choline acetyltransferase
Cpd E	Compound E
CAT7	Chromatin associated transcript 7
DI1	Delta-like 1
DI4	Delta-like 4
DMH1	a bone morphogenetic protein (BMP) inhibitor
EGF	Epidermal growth factor
ESCs	Embryonic stem cells
FGF	Fibroblast growth factor
FOXP1	Forkhead box protein 1
FGF2	Fibroblast growth factor 2
GNDF	Glial cell line-derived neurotrophic factor
GSK-3	Glycogen synthase kinase 3
hiPSCs	Human induced pluripotent stem cells
HOXC6	Homeobox C6
HST	Hoechst 33342
ISL1	Insulin gene enhancer 1
LHX3	LIM/homeobox 3
LHX1	Lim homeodomain transcription factors
lncRNAs	Long non-coding RNAs
miRNAs	microRNAs
MAP2	Microtubule Associated Protein 2
MEG3	Maternally expressed gene 3
MNs	Motor neurons
MS1	Musashi RNA Binding Protein
MYT1L	Myelin transcription factor 1 like
MX1/HB9	Motor neuron and pancreas homeobox 1
NPCs	Neural progenitor cells
NMJs	Neuromuscular junctions
DAPT	N-[N- (3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester
NKX2.2	NK2 homeobox 2
NT3	Neurotrophin-3
ncRNAs	Non-coding RNAs
NEAT1	Nuclear-enriched abundant transcript 1
NGN1	Neurogenin 1
NGN2	Neurogenin 2
NGN3	Neurogenin 3
NEUROD1	Neuronal Differentiation 1
NEUROD2	Neuronal Differentiation 2
Olig2	Oligodendrocyte transcription factor
OC1	Onecut transcription factors
PSA-NCAM	Polysialylated-neural cell adhesion molecule
POU5F1	POU class 5 homeobox 1
PHOX2A	Paired like homeobox 2A
POU3F2	POU class 3 homeobox 2
DLB	Dementia with Lewy bodies

PAX6	Paired box protein 6
PUR	Purmorphamine
qPCR	Quantitative PCR
RA	Retinoic acid signaling pathway
ROCK	Rho-associated coiled-coil containing protein kinase
Shh	Sonic Hedgehog signaling pathway
SOX2	Sex-determining region Y-box 2
SAG	Smoothed agonist
SOX1	SRY-Box Transcription Factor 1
SOX3	SRY-Box Transcription Factor
SOX11	SRY-box transcription factor 11
Synapsins	Regulation of neurotransmitter release at synapses
SYP	Synaptophysin
SMI-32	Neurofilament
TGFβ	Transforming growth factor-β
TUBB3	Tubulin Beta 3 Class III
TBX20	T-Box Transcription Factor 20
UTR	3'Untranslated region
VPA	Valproic Acid
VACHT	Vesicular acetylcholine transporter
WPI	Weeks post-viral infection
Waif1/5T4	Wnt-activated inhibitory factor 1
ZIC1	Zic family member 1

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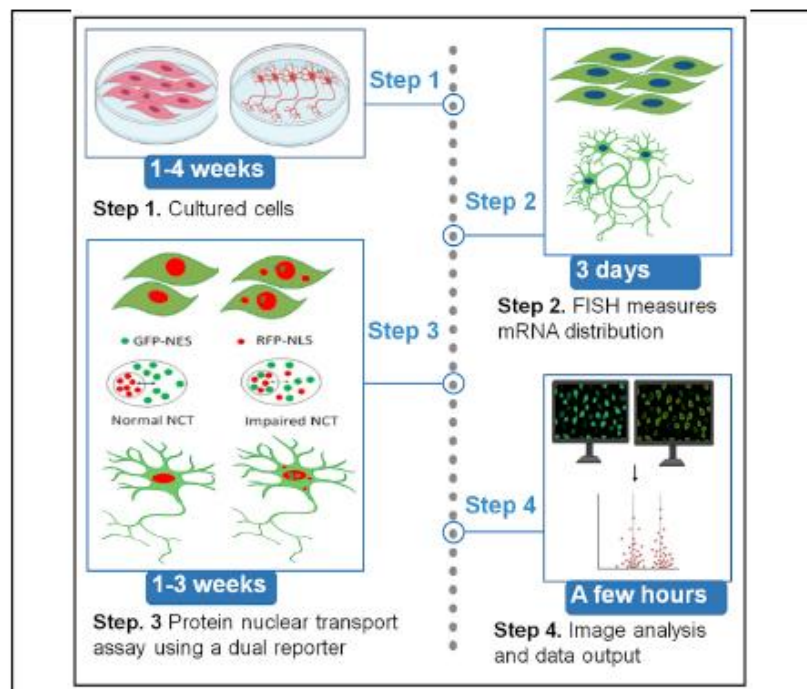
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STAR Protocols



Protocol

Protocol to image and quantify nucleocytoplasmic transport in cultured cells using fluorescent *in situ* hybridization and a dual reporter system



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Highlights

Measure nuclear
mRNA export by
fluorescent *in situ*
hybridization (FISH)

Examine protein
import and export
activities with a dual
reporter system

Use an open-source
software to quantify
images

Examine and quantify
NCT activities in
cultured cells,
including neurons

Nucleocytoplasmic transport (NCT) plays critical roles in maintaining cellular homeostasis. Here, we present a protocol to measure NCT for both transcript and protein cargos in cultured cells. We first describe the fluorescent *in situ* hybridization (FISH) assay to measure the nuclear mRNA export. We then detail a dual reporter system to measure the protein NCT. This protocol also includes image analysis and data output using CellProfiler™. The combined approach can be used to unbiasedly analyze NCT activities in cultured cells.

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

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Protocol

Protocol to image and quantify nucleocytoplasmic transport in cultured cells using fluorescent *in situ* hybridization and a dual reporter system

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SUMMARY

Nucleocytoplasmic transport (NCT) plays critical roles in maintaining cellular homeostasis. Here, we present a protocol to measure NCT for both transcript and protein cargos in cultured cells. We first describe the fluorescent *in situ* hybridization (FISH) assay to measure the nuclear mRNA export. We then detail a dual reporter system to measure the protein NCT. This protocol also includes image analysis and data output using CellProfiler™. The combined approach can be used to unbiasedly analyze NCT activities in cultured cells.

For complete details on the use and execution of this protocol, please refer to Ding et al. (2020, 2021).

BEFORE YOU BEGIN

Institutional permissions

1. Obtain the permission from Institutional Biosafety Committee for working on lentivirus in a BSL2 laboratory. This protocol has been approved and the BSL2 laboratory has been certified by the Institutional Biosafety Committee at LSU Health Sciences Center at Shreveport (Project number: B22-008).
2. Obtain the permission from institutional animal care and use committee (IACUC) for the animal study. This protocol has been approved by IACUC at LSU Health Sciences Center at Shreveport (Proposal number: P-22-037).

Preparation of research materials

3. Acquire all critical reagents mentioned in the [key resources table](#).
4. Acquire cell lines to be used in this protocol and prepare enough frozen stocks for the future research.
5. Acquire and prepare the high-quality lentiviral vectors to be used in this protocol.
6. Prepare media using recipes described in this protocol.

⚠ **CRITICAL:** All procedures are performed in a BSL-2 certified laboratory equipped with biosafety cabinet using standard aseptic techniques. Cultures are grown and maintained in a humidified incubator at 37°C with 5% CO₂.



KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Sheep anti-DIG, polyclonal (1:100)	MilliporeSigma	Cat# 11333089001, RRID: AB_514496
FITC conjugated Donkey anti-Sheep, polyclonal (1:200)	Abcam	Cat# ab6896, RRID: AB_955329
Rabbit anti-FITC, polyclonal (1:700)	Thermo Fisher Scientific	Cat# 71-1900, RRID: AB_2533978
Mouse anti-FITC, polyclonal (1:700)	Thermo Fisher Scientific	Cat# MA5-14696, RRID: AB_627695
Mouse anti-GFP, monoclonal (1:200)	Santa Cruz Biotechnology	Cat# sc-9996, RRID: AB_627695
Chicken anti-MAP2, polyclonal (1:10,000)	BioLegend	Cat# 822501, RRID: AB_2564858
Mouse anti-TUBB3 (1:3000)	Covance	Cat# MMS-435P, RRID: AB_2313773
Rabbit anti-TUBB3 (1:3000)	Covance	Cat# PRB-435P, RRID: AB_291637
Mouse anti-LMNA/C (1:500)	Abcam	Cat# ab40567, RRID: AB_775967
Alexa Fluor® 488- Donkey anti-Mouse IgG (1:250)	Jackson ImmunoResearch	Cat# 715-545-150, RRID: AB_2340846
Alexa Fluor® 488- Donkey anti-Rabbit IgG (1:250)	Jackson ImmunoResearch	Cat# 711-545-152, RRID: AB_2313584
Alexa Fluor® 594- Donkey anti-Rabbit IgG (1:250)	Jackson ImmunoResearch	Cat# 711-585-152, RRID: AB_2340621
Alexa Fluor® 594- Donkey anti-Chicken IgG (1:250)	Jackson ImmunoResearch	Cat# 703-585-155, RRID: AB_2340377
Alexa Fluor® 568- Donkey anti-Mouse IgG (1:250)	Thermo Fisher Scientific	Cat# A10037, RRID: AB_2534013
Bacterial and virus strains		
One Shot™ Stbl3™ Chemically Competent <i>E. coli</i>	Invitrogen	Cat# C737303
Experimental models: Cell lines		
Human embryonic kidney (HEK) 293T cells (passage number <20)	American Type Culture Collection (ATCC)	ATCC Cat# CRL-3216, RRID: CVCL_0063
Human fibroblast cells (passage number <15)	Coriell Institute For Medical Research	GM03211, RRID: CVCL_1U24; NDS00305; GM02304, RRID: CVCL_1U21; NDS00301; GM00024, RRID: CVCL_7269; Coriell Cat# GM03652, RRID: CVCL_7397; GM04506, RRID: CVCL_7413; AG07473, RRID: CVCL_2C33
Reprogrammed human neurons	(Ding et al., 2020, 2021; Sepehrmanesh et al., 2021; Sepehrmanesh and Ding, 2020)	N/A
Chemicals, peptides, and recombinant proteins		
Dulbecco's Modified Eagle Medium (DMEM)	Gibco	Cat# 11995065
Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM-F12)	HyClone	Cat# SH30023.02
Neurobasal Media	Thermo Fisher Scientific	Cat# 21103049
Fetal bovine serum (FBS)	Gibco	Cat# 16140071
Penicillin/Streptomycin (Pen/Strep)	Thermo Fisher Scientific	Cat# 15140122
N2 supplement (100x)	Thermo Fisher Scientific	Cat# 17502-048
B27 supplement (50x)	Thermo Fisher Scientific	Cat# 17504-044
Rho kinase (ROCK) inhibitor (Y-27632), 10 mM	MilliporeSigma	Cat# 129830-38-2
MEM Non-Essential Amino Acids Solution (NEAA)	Thermo Fisher Scientific	Cat# 11140050
GlutaMAX (100x)	Thermo Fisher Scientific	Cat# 35050
Brain Derived Neurotrophic Factor (BDNF)	PeptoTech	Cat# 450-02
Glial Derived Neurotrophic Factor (GDNF)	PeptoTech	Cat# 450-10
Neurotrophic factor 3 (NT3)	PeptoTech	Cat# 450-03
Heat Stable Recombinant Human Basic Fibroblast Growth Factor (bFGF)	Gibco	Cat# PHG0367
Human EGF recombinant protein	Gibco	Cat# PHG0311L
2-Mercaptoethanol (b-ME)	Thermo Fisher Scientific	Cat# 21985023

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Forskolin (FSK)	MilliporeSigma	Cat# F6886
Matrigel Matrix	Corning	Cat#356234
Gelatin	Thermo Fisher Scientific	Cat# G7-500
Trypsin	Thermo Fisher Scientific	Cat# 25200056
Nuclease-Free Water*	Thermo Fisher Scientific	Cat# AM9930
Ambion™ Recombinant RNase A*	Fisher	Cat# AM2269
Yeast tRNA*	Thermo Fisher Scientific	Cat# AM7119
Hoechst 33342 (HST)*	Thermo Fisher Scientific	Cat# 62249
Fisher Bioreagents™ Bovine serum albumin (BSA) DNase- and Protease-free Powder*	Thermo Fisher Scientific	Cat# BP9706100
Digoxigenin (DIG)-labeled oligo-dT (30 nt)*	Synthesized at IDT	N/A
Digoxigenin (DIG)-labeled oligo-dA (30 nt)*	Synthesized at IDT	N/A
Triton X-100 (TX-100)*	Thermo Fisher Scientific	Cat# 85111
Paraformaldehyde (PFA)*	Sigma-Aldrich	Cat# 158127
Formamide*	Sigma-Aldrich	Cat# FX0420-6
Ribonucleoside-vanadyl complex (RVC)*	New England Biolabs	Cat# S14025
Sodium citrate*	Thermo Fisher Scientific	Cat# BP327-1
Dextran sulfate*	Sigma-Aldrich	Cat# D-8906
ProLong Diamond Antifade Mountant*	Thermo Fisher Scientific	Cat# P36970
LB Broth	Thermo Fisher Scientific	Cat# 611875000
Ampicillin	Thermo Fisher Scientific	Cat# BP1760-5
Ethidium bromide solution	Roth	Cat# 2218.2
Polyethylenimine (PEI)	Polysciences	Cat# 23966-2
Leptomycin B (LMB)	Alfa Aesar	Cat# J63784
Recombinant DNA		
psPAX2	Addgene	RRID: Addgene_12259
pCMV-VSV-G	Addgene (Stewart et al., 2003)	RRID: Addgene_8454
pLVX-EF1alpha-2x GFP-NES-IRES-2x RFP-NLS (2G2R dual reporter)	Addgene (Mertens et al., 2015)	RRID: Addgene_71396
Critical commercial assays		
Plasmid DNA Midiprep Kits	Fisher	Cat# K0481
DNA gel loading dye, 6x	Thermo Scientific™	Cat# R0611
GeneRuler 1 kb DNA ladder	Thermo Scientific™	Cat# SM0312
DNase/RNase-free water	Invitrogen	Cat# 10977049
Software and algorithms		
ImageJ	NIH (Schneider et al., 2012)	ImageJ RRID: SCR_003070
CellProfiler	Broad Institute of MIT and Harvard	CellProfiler RRID: SCR_007358
Other		
Laminar Culture Hood	Thermo Fisher Scientific	Cat# SHKE435HP
CO ₂ Incubator	VWR	Cat# 10810-888
Shaker incubator	Thermo Fisher Scientific	Cat# SHKE436HP
Microbiological Incubator	Thermo Fisher Scientific	Cat# 50125590
Centrifuge 5420	VWR	Cat# 76404-386
Centrifuge 5418R	VWR	Cat# 97058-928
Centrifuge MEGAFUG	VWR	Cat# 89511-734
NanoDrop	Thermo Fisher Scientific	Cat # ND-2000
ChemiDoc™ Touch Imaging System	Bio-Rad	Cat# 1708370
Freezer –20°C	Fisher Scientific	FBV20FPSA
Freezer –80°C	VWR	Cat# 76318-814
100 mm tissue culture disc	Thermo Scientific	Cat# 130182
Microscope slides	Fisher Scientific	Cat# 12-550-15
6-well plate	Corning	Cat# 490007-412
12-well plate	Corning	Cat# 83-3336
24-well cell culture plates	Thermo Fisher Scientific	Cat# 142485
Fisherbrand™ Petri Dishes	Thermo Fisher Scientific	Cat# FB0875713

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
0.45-mm vacuum filter	Millipore	Cat# SLHV033RB
0.22-mm vacuum filter	Millipore	Cat# SLGV033RS
12-mm cover slips	Fisherbrand	Cat# 12-545-80P
Microscope slides	Fisher Scientific	Cat# 12-550-15
Falcon 50 mL Conical tube	Corning	Cat# 14-432-22
Falcon 15 mL Conical tube	Corning	Cat# 14-959-53A
Premium Microcentrifuge Tubes: 1.5 mL	Fisherbrand	Cat# 05-408-129
Eppendorf adjustable-volume pipette, P20	Thermo Fisher Scientific	Cat# 13-690-027
Eppendorf adjustable-volume pipette, P200	Thermo Fisher Scientific	Cat# 13-690-030
Eppendorf adjustable-volume pipette, P1000	Thermo Fisher Scientific	Cat# 13-690-032
Filter tips,	Fisher Scientific	Cat# 10394121/2139
Filter tips, 20 μ L	Fisher Scientific	Cat# 10282761
Filter tips, 200 μ L	Fisher Scientific	Cat# 10029040
Filter tips, 1000 μ L	Fisher Scientific	Cat# 10045460
Pipette Controller	Thermo Fisher Scientific	Cat# TS 9501
5 mL disposable polystyrene serological pipettes	Thermo Fisher Scientific	Cat# 13-675-22
10 mL disposable polystyrene serological pipettes	Thermo Fisher Scientific	Cat# 13-675-20
25 mL Disposable polystyrene serological pipettes	Thermo Fisher Scientific	Cat# 13-675-30
Spray bottle containing 70% (vol/vol) ethanol	N/A	N/A
Microscope matched computer equipped with imaging system	Dell	Precision 3450
Inverted Fluorescence Microscope	Olympus	Cat# CKX53F3
Leica TCS SP5 Confocal microscope	Leica	RRID: SCR_020233
Leica DMI 6000 CS Inverted Confocal Microscope	Leica	RRID: SCR_020220

*Reagents for FISH assay.

MATERIALS AND EQUIPMENT

20 $\times$ saline-sodium citrate (SSC) solution

Reagents	Final concentration	Amount
NaCl	3 M	175.3 g
Sodium citrate $\cdot$ 2H ₂ O	300 mM	88.2 g
Citric acid	N/A	
Nuclease-free water	N/A	~1,000 mL
Total	N/A	1,000 mL

⚠ **CRITICAL:** Adjust pH to 5.0 with citric acid and make volume up to 1,000 mL with nuclease-free water. The 20 $\times$ SSC solution should be stored at RT for up to 2 years. For making 4 $\times$, 2 $\times$, and 1 $\times$ SSC, just dilute 20 $\times$ SSC with nuclease-free water as 1:5, 1:10, and 1:20, respectively.

Formamide solution

Reagents	Final concentration	Amount
Formamide	20%	200 μ L
4 $\times$ SSC	2 $\times$	500 μ L
Nuclease free water	N/A	300 μ L
Total	N/A	1,000 μ L



⚠ **CRITICAL:** Aliquot and store stocks of formamide in -80°C for up to 2 years. If it is liquefied at -20°C , deionization is required. Use a single stock aliquot to prepare 20% formamide solution freshly in the day of use.

Note: Formamide is carcinogenic and need to wear protective clothing and mask.

Hybridization buffer (2×HB in 4×SSC)		
Reagents	Final concentration	Amount
Dextran sulphate	120 mM	2 g
Ribonucleoside Vanadyl Complex (RVC)	20 mM	1 mL
20× SSC	4×	2 mL
Nuclease-free water	N/A	~ 7 mL
Total	N/A	10 mL

⚠ **CRITICAL:** Use a 50 mL conical tube to add 2 g of dextran sulfate and then add 5 mL of nuclease-free water slowly. Heat the mixture in a 37°C water bath for 30 min. Add 2 mL of 20× SSC and then 1 mL of RVC and continue incubation in a 37°C water bath to become light gray. Then make volume up to 10 mL with nuclease-free water. Store at -80°C for up to 1 year.

Note: RVC is irritating. Users need to wear safety goggles and protect skin and body by wearing appropriate protective cloths.

1× HB without probe		
Reagents	Final concentration	Amount
2× HB	1×	10 mL
Formamide	20%	4 mL
Nuclease free water	N/A	~ 6 mL
Total	N/A	20 mL

Note: This solution is used to briefly equilibrate samples before hybridization with probes. It needs to be made fresh.

1× HB with probe (Oligo-dT working solution)		
Reagents	Final concentration	Amount
2× HB	1×	10 mL
Formamide	20%	4 mL
Oligo-dT (1000×	1×	20 μL
Nuclease free water	N/A	~ 6 mL
Total	N/A	20 mL

⚠ **CRITICAL:** Heat a 50 mL conical tube contain ~6 mL nuclease-free water and 4 mL of formamide at 100°C , indirectly. After adding oligo-dT, heat for 10 min at 100°C and then put on ice. After cooling, add 10 mL of 2×HB and aliquot and store in -80°C for up to 1 year.

Note: Formamide is carcinogenic and need to wear protective clothing and mask.

Sheep anti-DIG solution

Reagents	Final concentration	Amount
Ribonucleoside Vanadyl Complex (RVC)	N/A	80 μ L
4x SSC	N/A	800 μ L
Sheep anti-DIG, polyclonal (1:100)	N/A	20 μ L
Total	N/A	900 μ L

⚠ **CRITICAL:** After adding RVC to 4x SSC, heat the solution in in 37°C water bath till gray. Then add antibody. Scale up and down according to samples. Recycled Sheep anti-DIG can be stored in –80°C for up to 1 year and reused directly but do not reuse more than 3 times because the signals will be gradually diminished.

Note: RVC is irritating and need to wear safety goggles and protect skin and body by wearing appropriate protective cloths.

Blocking buffer

Reagents	Final concentration	Amount
BSA	30 mg/mL	300 mg
Triton X-100	0.1%	10 μ L
1x PBS	N/A	~10 mL
Total	N/A	10 mL

⚠ **CRITICAL:** It is better to prepare freshly. You can use this as blocking buffer and diluting solution for primary and secondary antibodies.

HEK293T culture medium

Reagents	Final concentration	Amount
Dulbecco's Modified Eagle Medium (DMEM)	N/A	45 mL
Fetal bovine serum (FBS)	10%	5 mL
Pen/ Strep (100x)	1x	500 μ L
Total	N/A	~50 mL

⚠ **CRITICAL:** Always prepare freshly before use. Store complete medium at 2°C–8°C for up to 2 weeks.

Neuronal maturation medium

Reagents	Final concentration	Amount
DMEM:F12	N/A	32 mL
Neurobasal	N/A	16 mL
Pen/Strep (100x)	1x	500 μ L
N2 (100x)	1x	500 μ L
B27 (50x)	1x	1 mL
FSK (20 mM)	5 μ M	12.5 μ L
BDNF (20 μ g/mL)	10 ng/mL	25 μ L
GDNF (20 μ g/mL)	10 ng/mL	25 μ L
NT3 (20 μ g/mL)	10 ng/mL	25 μ L
Total	N/A	~50 mL

⚠ **CRITICAL:** Always prepare freshly before use. Store complete medium at 2°C–8°C for up to 2 weeks.



Neural progenitor cell medium (NPC Medium)		
Reagents	Final concentration	Amount
DMEM:F12	N/A	32 mL
Neurobasal	N/A	16 mL
Pen/Strep (100×)	1×	500 μ L
N2 (100×)	1×	500 μ L
B27 (50×)	1×	1 mL
Glutamax (100×)	1×	500 μ L
NEAA (100×)	1×	500 μ L
b-ME (50 mM, 1000×)	50 μ M	50 μ L
bFGF (200 μ g/mL)	10 ng/mL	2.5 μ L
EGF (100 μ g/mL)	10 ng/mL	5 μ L
Rock inhibitor (Y-27632, 10 mM)	10 μ M	50 μ L
Total	N/A	~50 mL

⚠ **CRITICAL:** Always prepare freshly before use. Store complete medium at 2°C–8°C for up to 2 weeks.

Leptomycin B stock		
Reagents	Final concentration	Amount
HEK293T culture medium	N/A	50 mL
Leptomycin B (1 mM)	50 nM	2.5 μ L
Total	N/A	~50 mL

⚠ **CRITICAL:** Always prepare freshly. The medium is ready to add to cells.

PEI solution stock		
Reagents	Final concentration	Amount
PEI	1 μ g/ μ L	50 mg
MilliQ water	N/A	50 mL
Total	N/A	~50 mL

⚠ **CRITICAL:** To facilitate dissolving PEI, use autoclaved nuclease-free water with pH ~2 (adjusted with HCl). Stir until all the PEI dissolved, adjust pH to 7 using 2 N NaOH. Filter the solution through a 0.22 μ m filter. Aliquot 1 mL of PEI solution into each 1.5 mL microtubes. Store them in –80°C for up to 2 years.

Note: Inhalation of PEI may cause irritation in respiratory tract. Use a fume hood.

Transfection mixture		
Reagents	Final concentration	Amount
DMEM	N/A	10 mL
PEI stock solution (1 μ g/ μ L)	4 μ g/mL	40 μ L
2Gi2R dual reporter	1 μ g/mL	10 μ L
Total	N/A	~10 mL

⚠ **CRITICAL:** Add PEI at last step, and mix it well by vortexing or quickly pipetting. Scale up and down according to the total amount of cells to be transfected.

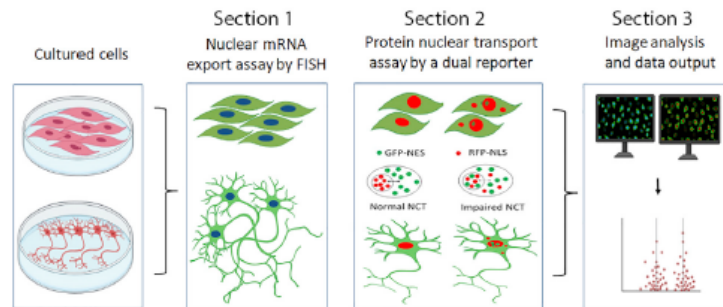


Figure 1. Schematic shows the major steps of this protocol

The starting materials are cultured cells (e.g., fibroblast cells and neurons). **Section 1: nuclear mRNA export measurement by FISH assay**, nuclear mRNA export measurement by the fluorescent *in situ* hybridization (FISH) assay. **Section 2: protein NCT measurement by a dual reporter system**, protein NCT assay by a dual reporter, green fluorescent protein fused with the nuclear export sequence (GFP-NES) and red fluorescent protein fused with the nuclear localization sequence (RFP-NLS). **Section 3: image analysis using CellProfiler software**, confocal image analysis followed by data output using CellProfiler™ Software.

STEP-BY-STEP METHOD DETAILS

This protocol starts with cultured cells and focuses on three sections, 1) the fluorescent *in situ* hybridization (FISH) assay to measure the nuclear mRNA export, 2) a dual reporter system to measure the protein NCT, and 3) image analysis and data output using CellProfiler™ Software (Figure 1). For details on cell culture and reprogramming of human neurons, please refer to our previous publications (Akte et al., 2022; Ding et al., 2020, 2021; Sepehrmanesh et al., 2021; Sepehrmanesh and Ding, 2020).

Section 1: Nuclear mRNA export measurement by FISH assay

⌚ Timing: 3 days

This section describes the protocol of measuring nuclear mRNA export using the FISH assay with oligo-dT probes, which specifically bind the poly(A) tails of mRNAs. Importantly, the FISH assay could be combined with immunocytochemistry (ICC) for detecting specific markers, which will be used to define the nucleus and the cytoplasm of target cells. This experiment consists of three major steps. 1) Cultured cells on coverslips are fixed and then hybridized with Digoxigenin (DIG)-labeled oligo-dT probes. 2) Following the standard ICC, mRNA signals are to be detected using the anti-DIG antibody, and the nucleus and/or cell identity markers will be co-stained with other compatible antibodies or dyes. 3) Based on confocal images, the subcellular distributions of nuclear and cytoplasmic fractions for total mRNAs will be analyzed, and the nuclear mRNA export activity will be evaluated by the ratio of the oligo-dT signal in the nucleus to the cytoplasm. The higher ratio of $dT_{(Nuc/Cyt)}$ indicates compromised nuclear mRNA export.

Day 1. *In situ* hybridization

⌚ Timing: 2–4 h

In this step, DIG-labeled oligo-dT is used to probe mRNAs. To detect specific transcripts, the probes could be designed with the complementary sequence to the target RNAs and labeled with DIG. The following procedure is described based on cultured cells on coverslips in a 24-well plate.

1. Set up *in situ* hybridization to detect mRNAs in cultured cells.



- a. Remove the culture media and briefly wash once with ~250 μ L per well of 1 $\times$ PBS.
- b. Add 250 μ L of 4% paraformaldehyde (PFA) per well and fix the cells at room temperature (RT) for 30 min.

Note: The volume of 250 μ L is based on the 24-well plate. Please adjust the volume if you use different culture plates or containers to perform this assay.

Note: If samples cannot be processed immediately, replace PFA with 500 μ L per well of 70% ethanol (diluted with the nuclease-free water). Seal the plate well to avoid drying out. It is not harmful to keep coverslips at -20°C for a few weeks without an obvious decrease in oligo-dT signals. Once you are ready, continue the process from step c).

- c. Remove PFA (or alternatively 70% ethanol from step b), wash with 1 $\times$ PBS three times, each time 250 μ L per well for 5 min.
- d. For each well, add 250 μ L of 1 $\times$ HB without probe and incubate for 3 min at 37°C to equilibrate samples.
- e. Remove 1 $\times$ HB without probe, add 250 μ L of hybridization solution (1 $\times$ HB with probe) that contains oligo-dT (or oligo-dA for negative control), yeast tRNA (to minimize the non-specific binding), and 20% formamide in 1 $\times$ HB.
- f. Incubate overnight (10–16 h) at 37°C .

Note: It is better to keep plates in a wet chamber during incubation to avoid drying out.

Day 2. ICC to detect probes and specific cellular markers

⌚ Timing: 8–10 h

In this step, antibodies are added sequentially and washed to detect oligo-dT probes. Some steps need to be performed at 37°C and others are performed at RT. The use of fresh buffer and high-quality antibodies in this step is very important.

2. To detect probe and cellular markers.
 - a. Thaw a formamide aliquot from -80°C to prepare a proper volume of wash buffer containing 20% formamide in 2 $\times$ SSC.
 - b. Remove hybridization solution.

Note: Hybridization solution can be reused for up to 3 times.

- c. Add 250 μ L of 2 $\times$ SSC containing 20% formamide and incubate for 15 min at 37°C .

Note: For each wash step, carefully add/remove liquid from the edge of a well to avoid detaching cells from coverslips.

- d. Wash with 250 μ L of 2 $\times$ SSC per well for 15 min at 37°C .
- e. Remove 2 $\times$ SSC and wash with 250 μ L of 1 $\times$ SSC per well for 15 min at 37°C .
- f. Prepare antibody mixture containing Sheep anti-DIG and RVC in 4 $\times$ SSC.
- g. Remove 1 $\times$ SSC, add 200 μ L of Sheep anti-DIG solution (1:100) and incubate for 2 h at 37°C .
- h. Remove (and alternatively recycle) the Sheep anti-DIG solution.

Note: After this step, the 37°C incubator is not required, and all the following steps will be performed at RT.

- i. Wash with 250 μ L of 4 $\times$ SSC per well for 10 min at RT.
- j. Remove 4 $\times$ SSC and wash with 250 μ L of 4 $\times$ SSC containing 0.1% Triton X-100 for 10 min at RT.
- k. Wash again with 250 μ L of 4 $\times$ SSC per well for 10 min at RT.
- l. Remove 4 $\times$ SSC and fix cells with 250 μ L of 4% PFA per well for 10 min at RT.
- m. Remove fixing buffer and wash with 250 μ L of freshly prepared permeabilization buffer 1 $\times$ PBS containing 0.1% Triton X-100 (0.1% PBS-T) for 10 min at RT.

Note: The following steps are the same as the standard ICC protocol (Ding et al., 2013, 2016, 2018).

- n. Remove permeabilization buffer and wash with 250 μ L of 1 $\times$ PBS for 10 min at RT.
- o. Remove 1 $\times$ PBS and add the FITC-labeled Donkey anti-Sheep antibody (1:200) and incubate for 2 h at RT.

Note: The FITC-labeled antibody is light sensitive and thus samples need to be kept from light in the following steps, e.g., by covering a plate with an aluminum foil.

- p. Remove the antibody solution and wash with 1 $\times$ PBS three times at RT, 10 min for each time.

Note: After this step, coverslips could be mounted on a glass slide and oligo-dT signals could be visualized based on FITC. Since the FITC signal is usually weak, perform the following steps to amplify the signal:

- q. Remove 1 $\times$ PBS and incubate samples with 250 μ L of blocking buffer (3% BSA in 1 $\times$ PBS) for 10 min at RT.
- r. Add Rabbit anti-FITC antibody (1:700) and Chicken anti-MAP2 (1:10,000), and incubate overnight (10–16 h) at 4°C.

Note: Antibodies should be diluted in 3% BSA in 0.1% PBST to minimize non-specific binding.

Note: Use different combinations of antibodies for specific samples to distinguish mRNA signals and other protein markers with different colors. If you will use a new secondary antibody that the specificity has not been verified, you need to set up a secondary antibody control.

Day 3. mount coverslips and visualization

⌚ **Timing: 4–6 h**

3. This step describes how to visualize mRNA probed with a primary antibody using fluorescent conjugated secondary antibodies. Using secondary antibodies with different colors to distinguish the oligo-dT signals and specific protein markers, imaging is recommended to be performed under a confocal microscope.
 - a. Remove primary antibodies and wash four times with 250 μ L of 1 $\times$ PBS at RT, each time for 10 min.
 - b. Remove 1 $\times$ PBS of the last wash, add corresponding secondary antibodies, and incubate for 2 h at RT in dark. Here, we used AF488 Donkey anti-Rabbit (1:250) and AF594 Donkey anti-Chicken (1:250).

Note: Antibodies should be diluted in 3% BSA in 0.1% PBS-T to minimize non-specific binding.



Note: Usually, we use Alexa Fluor 488 for dT signals since it is brighter. If samples contain a GFP reporter, then use Alexa Fluor 594 for dT signals.

- c. Remove the secondary antibody solution and wash three times with 250 μ L of 1 $\times$ PBS at RT, and each time for 10 min.
- d. Remove 1 $\times$ PBS from the last wash. Add the 250 μ L of nucleic dye HST or DAPI (1 μ g/mL in PBS) and incubate for 5 min at RT.
- e. Remove HST solution and wash with 250 μ L of 1 $\times$ PBS for 2 min at RT.
- f. Briefly wash with 250 μ L of nuclease-free water for 1 min at RT.
- g. Air dry coverslips at RT (in dark) for 20 min or use a paper towel to carefully remove liquid from the edges of coverslips.
- h. Mount coverslips on slides with a mounting medium. Allow mounting medium to be fully hardened at RT in dark (about 30 min to a few hours).

Note: To preserve fluorescent signals, the slides should be kept at 4°C for short-term storage or at –20°C for long-term storage. If the mounting medium is not fully hardened, the cells could be detached from coverslip when visualizing samples.

- i. Visualize the FISH samples under a confocal microscope.

Note: In this protocol, we used the Leica DMI 6000 CS Inverted Confocal Microscope with motorized stage, condenser, objective, and filter turrets. The objectives were used are 40 $\times$ air lens (HCX PL APO CS 40 $\times$ /0.75U-V-I 0.17) or 63 $\times$ oil lens (HCX PL APO CS 63 $\times$ /1.4–0.6NA) and the resolution of images is 1024 $\times$ 1024 pixels. Any equivalent confocal microscope with similar settings should be good to produce confocal images for later quantification analysis.

- j. Confocal image analysis (see [section 3: image analysis using CellProfiler software](#) of this protocol).

Section 2: Protein NCT measurement by a dual reporter system

⌚ Timing: 4 weeks

In this section, we will describe the procedure to examine protein nuclear transport using a dual reporter system, in which GFP-NES and RFP-NLS are co-expressed ([Mertens et al., 2015](#)). Therefore, both protein export and import activities can be analyzed in individual cells. Direct transfection of a reporter plasmid could be employed for rapid-growing cells, such as HEK cells. For slow-growing postmitotic cells, such as primary and reprogrammed neurons, it is required to prepare appropriate lentivirus to deliver the reporter vector. To achieve high transduction efficiency and the proper expression of the dual reporter, it is critical to prepare a high-quality lentivirus.

This part consists of five major steps. 1) The dual reporter plasmid preparation, including the transformation of a plasmid into Stbl3 competent cells and plasmid midi preparation. 2) The validation of the dual reporter system in HEK cells with the treatment of Leptomycin B (LMB), a specific nuclear export inhibitor ([Jang et al., 2003](#)). 3) The dual reporter lentivirus preparation. This dual reporter system was constructed with the second-generation lentiviral vector. 4) Transduction of the dual reporter lentivirus to fibroblast cells or cultured neurons. 5) Dual reporter analysis. Depending on cell subtypes and the developmental stages to be analyzed, infected cells could be visualized at 1–4 wpi (weeks post-viral infection). Once the reporter system has been validated, only repeating steps 3–5 to measure the protein transport in cultured cells are needed.

Preparation of a lentiviral vector

⌚ **Timing:** 3–5 h

4. This step describes the purification of a lentiviral vector via plasmid Midi prep columns.
 - a. Transform of a lentiviral vector to Stbl3™ chemical competent cells (or equivalent) and culture them in 50 mL of LB medium with 100 µg/mL Ampicillin.

Note: For lentiviral plasmid preparation, the culture temperature between 30°C–35°C is better than 37°C in the maintenance of plasmid stability. The culture time to reach saturation varies according to the temperature, from overnight (10–16 h) to 30 h.

- b. The next morning, collect the bacteria at 1,000 × g for 5 min.
 - c. Purify the plasmid via Midi prep column according to the manufacturer's instructions. For detailed information, please see [User Guide: Plasmid Midiprep Kit \(thermofisher.com\)](#).
 - d. Examine the plasmid concentration by using a NanoDrop and evaluate the plasmid quality by running an agarose gel.

Note: A good yield of a plasmid midi prep is no less than 100 µg from a 50 mL culture. The quality of newly purified plasmids could be evaluated by the ratio of the absorbance at 260 and 280 nm ($A_{260}/A_{280} \geq 1.8$) and by running an agarose gel. The desired plasmid DNA is the supercoiled form, which migrates faster than predicted (based on size) in an agarose gel due to its conformation. The major band on the agarose gel should be the supercoiled form.

The validation of the reporter system

⌚ **Timing:** 7 days

5. In this step, the dual reporter system is validated in HEK cells with the treatment of Leptomycin B (LMB), a specific nuclear export inhibitor ([Jang et al., 2003](#)). In the presence of LMB, nuclear export of GFP-NES will be disrupted, while the distribution of RFP-NLS won't be influenced.
 - a. Culture HEK cells in DMEM containing 10% FBS and 1 × Pen/Strep in a CO₂ incubator at 37°C.
 - b. When cells reach about 80% confluency (~2 or 3 days), collect cells by trypsinization with 0.05% Trypsin in PBS.
 - c. Seed HEK cells onto gelatin coated coverslips in a 12-well plate at the cell density of about 5×10^4 cells/cm². After seeding, shake a plate well to reach homogenous cell density.

Note: For coating coverslips, incubate coverslips with 0.2% gelatin at 37°C for more than 30 min.

- d. The next day, HEK cells were transfected with reporter plasmid using PEI solution. For each well of the 12-well plate, add 1 µg plasmid and 4 µg PEI to 50 µL of serum-free DMEM.

Note: It is better to prepare the transfection mixture for all wells together.

- e. Immediately mix the transfection mixture after the addition of PEI by either vortexing for 15 s or quickly pipetting up and down five times.
 - f. Incubate the transfection mixture for 15 min at RT.
 - g. Add 50 µL of transfection mixture to each well of the 12-well plate and put it back into a CO₂ incubator.
 - h. The next day, change the whole volume of HEK culture medium (1 mL/well).



Note: 48–72 h post-transfection, distinct GFP and RFP signals can be noticed under a fluorescent microscope. Treat cells with Leptomycin B (LMB) as follows to validate the dual reporter system:

- i. Prepare a culture medium that contains 50 nM LMB.
- j. Fully change the culture medium containing 50 nM LMB (1 mL/well).
- k. Take coverslips at 0-, 30-, 60- and 90-min post-exposure to LMB and directly mount them on slides.
- l. Visualize the cells by confocal microscope.
- m. Confocal micrographs will be analyzed by CellProfiler software as described in [section 3: image analysis using CellProfiler software](#) of this protocol.

Note: The effectiveness of the dual reporter system can be demonstrated by the gradual accumulation of nuclear GFP signal, while the distribution of RFP will be not influenced.

Lentivirus preparation

⌚ **Timing: 4 days**

6. Preparation of lentivirus using HEK293T cells.

Note: To examine the protein transport in post-mitotic cells such as cultured neurons, preparation of lentivirus expressing the dual reporters is necessary. This dual reporter system was constructed with the second-generation lentiviral vector. Two extra helper plasmids (psPAX2 and pCMV-VSVG) are required for the assembly of the viral particles. For more information about lentivirus preparation using the second-generation system, please refer to our previous report ([Ding and Kilpatrick, 2013](#)).

- a. Culture HEK293T cells as described in step 5.
- b. For transfection, seed about 8×10^6 HEK cells in each 10 cm plate in a 10 mL HEK culture medium.

Note: The next day, HEK cells should be around 80% confluency before transfection.

- c. Prepare the lentivirus transfection mixture in a 15 mL conical tube according to the recipe.
- d. For each 10 cm plate, add 15 μ g of a dual reporter plasmid, 10 μ g of psPAX2 and 5 μ g of pCMV-VSVG to 1 mL serum-free DMEM and mix well.

Note: Scale up if more than one plate will be prepared.

- e. Add 90 μ g of PEI (90 μ L of 1 μ g/ μ L PEI stock) and immediately mix by vortexing for 15 s.
- f. Leave the transfection mixture for 15 min at RT.
- g. Gently add 1 mL transfection mixture to each 10 cm plate and mix well to distribute it evenly.
- h. Incubate cells in a 37°C incubator.
- i. After overnight (~16 h) exposure to transfection reagents, fully change the HEK culture medium.
- j. Collect viral supernatant at 24 h post medium change and add back 10 mL of the HEK culture medium to each plate. Put the plates back into the incubator and store the viral supernatant at 4°C.
- k. After another 24 h, collect the viral supernatant for the second time and discard the plate in a biohazard bag.

Note: All lentivirus-contaminated materials (e.g., plates and pipettes) will be trashed in a container labeled with biohazard and processed according to the institutional biosafety protocol. Any spill of the lentivirus in the biosafety hood or other devices needs to be immediately wiped with 70% ethanol or 10% bleach.

- l. Pool these two collections together. Filter the viral supernatants with 0.45 μ m vacuum filters.
- m. Store viral supernatants at 4°C until the immediate use within two weeks or freeze them at –80°C for long-term storage.

Note: The titer of the virus could be estimated by the transduction of HEK cells with a series of dilutions of the virus as previously reported (Ding and Kilpatrick, 2013). A good titer of lentiviral supernatants should be no less than 1×10^6 IFU/mL. (IFU, Infectious Unit).

Transduction of the lentivirus to cultured cells

⌚ **Timing: 2 days**

7. This step describes the transduction of the dual reporter lentivirus to human fibroblast cells or cultured neurons.

⚠ CRITICAL: To achieve a reasonable infection efficiency, the dosage of the virus should be optimized to transduce different cell types. Too much virus may cause more cell death.

Note: The transduction efficiency and the cell survival should be well balanced. Generally, about 60% transduction efficiency is good enough to examine the protein transport in most samples. We usually employ 5 MOI (multiplicity of infection) to infect fibroblast cells, and around 2 MOI to transduce primary or iPSC-derived neural progenitor cells (NPCs). For transduction of post-mitotic and differentiated neurons, we suggest using 3 or 4 MOIs.

Note: In this step, iPSC-derived NPCs are taken as an example to show the procedure of transduction and examination of protein NCT using the dual reporter system. For more information about NPC generation and induction of specific neuronal subtypes, please refer to (Akter et al., 2022; Ding, 2021; Ding et al., 2021; Sepehrmanesh and Ding, 2020).

- a. Prepare a Matrigel-coated 6-well culture plate and incubate it for more than 30 min at 37°C.

Note: For detailed to prepare Matrigel-coated plates, please refer to our previous report (Akter et al., 2022).

- b. Quick thaw an NPC frozen stock in a 37°C water bath and transfer cells into a 15 mL tube containing 5 mL of warm NPC medium.
- c. Spin down cells at 200 $\times$ g for 3 min.

Note: For one frozen stock with 1×10^6 cells, seed onto 3 wells of a 6-well plate.

- d. Gently resuspend cell pellets into a proper volume of NPC medium and seed NPCs onto Matrigel-coated plates at a density of 3×10^4 cells/cm².
- e. When NPCs reach ~60% confluence, add the proper amount of lentivirus to each well.

Note: For lentivirus transduction, the proper NPC density (50%–60% confluence) is critical to obtain neurons with high yield and purity.



- f. NPCs are exposed to lentivirus for about 6 h to overnight (10–16 h). Replace the culture medium with Neuronal Maturation Medium.
- g. Change the half volume of Neuronal Maturation Medium every other day till analysis or replating.

Note: The age of induced neurons (iNs) is counted from the lentiviral transduction as dpi (day post-viral infection). NPCs become neuron-like in morphology after 3 dpi.

Note: Both GFP and RFP signals can be noticed at 3 dpi and the signal density increases gradually.

- h. The protein transport could be analyzed by imaging living cells under an inverted fluorescence microscope with the focus on dual reporter positive cells at desired time points.
- i. To analyze the protein transport in mature neurons (more than 14 dpi), replat neurons onto coverslips with a monolayer of astrocytes at 5 dpi as previously reported (Akter et al., 2022). Change the half volume of Neuronal Maturation Medium every other day till analysis.
- j. At desired time point, take the coverslips from the culture plate.
- k. Incubate coverslips with Hoechst 33342 (HST) (1 μ g/mL in PBS) for 10 min at RT.
- l. Briefly rinse the coverslips twice with PBS and mount the coverslips on slides.
- m. Image samples with a confocal microscope.
- n. Confocal micrographs will be analyzed by CellProfiler software as described in [section 3: image analysis using CellProfiler software](#) of this protocol.
- o. The ratio of nuclear to cytoplasmic GFP_(Nuc/Cyt) will be used to evaluate protein export, and the ratio of cytoplasmic to nuclear RFP_(Cyt/Nuc) will be used to evaluate protein nuclear import.

Section 3: Image analysis using CellProfiler software

⌚ Timing: hours to days

Confocal images of FISH assay or reporter assay could be analyzed using software such as ImageJ or CellProfiler™. At single cell levels, the subcellular distributions of dT signal or fluorescent protein levels will be measured. For each cell, as large an area as possible was measured within the nucleus or cytoplasm. An unbiased approach to data collection is recommended (Ding et al., 2020). For example, the individual performing the analysis using CellProfiler is completely blinded to sample information.

Images of nuclear markers are needed to properly designate nuclear boundaries before beginning. Additionally, images of the stained translocated objects of interest are needed. Overlaying images are not required for quantification but can be used as controls to ensure the correct subcellular areas are selected for the measurement. The mean values are used to quantify an average signal density. For FISH assays, the average of oligo-dT signals in the soma (both nucleus and cytoplasm) was used to evaluate the overall mRNA expression levels. Nuclear mRNA export was measured by the ratio of nuclear to cytoplasmic oligo-dT signals (dT_(Nuc/Cyt)). For the dual reporter assay, GFP and RFP signals will be separately measured in the nucleus and cytoplasm. The ratio of nuclear to cytoplasmic GFP_(Nuc/Cyt) is used to evaluate the protein export activity, while the ratio of cytoplasmic to nuclear RFP_(Cyt/Nuc) is used to evaluate protein import activity. In this section, the confocal images of the FISH assay in fibroblast cells are used as an example to demonstrate the procedure of quantification.

Import confocal images to CellProfiler

⌚ Timing: 5–10 min

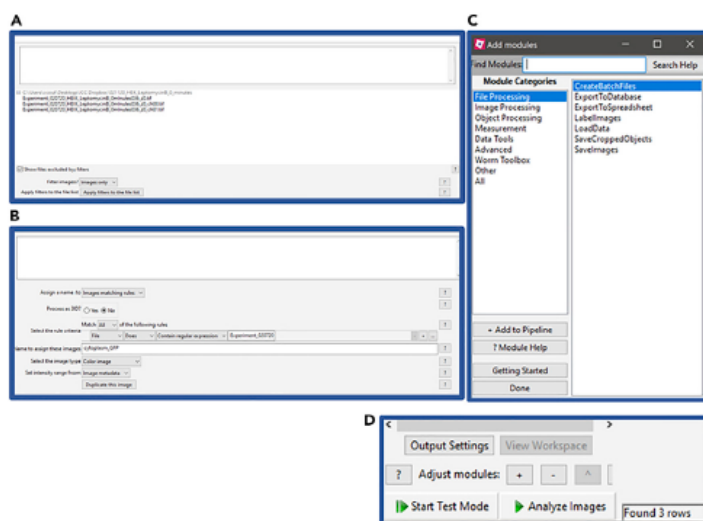


Figure 2. Importing images

(A) CellProfiler™ Images module for inputting images for processing.
(B) CellProfiler™ NamesAndTypes module for designating images for processing.
(C and D) CellProfiler™ module insert, test mode, and analyze images options. Insert numbers or letters to designate options.

8. This step describes the import of confocal images using CellProfiler software.
 - a. Upload images to be analyzed into the CellProfiler™ application images tab. This can be accomplished by dragging and dropping files into the designated box for files (Figure 2).

Note: Take note of the image names, as they will serve as initial tags for the files.

- b. Under the NameAndTypes tab assign names to image matching rules. This allows the assignment of different names to images with specific criteria/alterations in downstream applications.
- c. As the images are 2-dimensional, select no for “Process as 3D”.
- d. Select “All” for matching rules of the image.
- e. Select the rule criteria: This option states what the image contains. I.e., if the image contains images of chromatin staining, you will want to enter the following settings: “File” “Does” “Contain regular expression” “Title of photo uploaded that stains for chromatin”.
- f. Enter the name you wish to assign to the image.
- g. For this experiment, color images are uploaded before converting them to grayscale. Because of this, you will set “Select the image type” as “Color image”
- h. Select “Image metadata” for “Set intensity range from”. This will rescale fluorescence intensities for oversaturated areas of the image.
- i. Repeat the previous steps to include additional needed images such as overlaid images or other stained targets. At any time, you may enter test mode to run portions of your pipeline to ensure images are being processed properly.

Convert color images to grayscale

⌚ **Timing:** 5–10 min

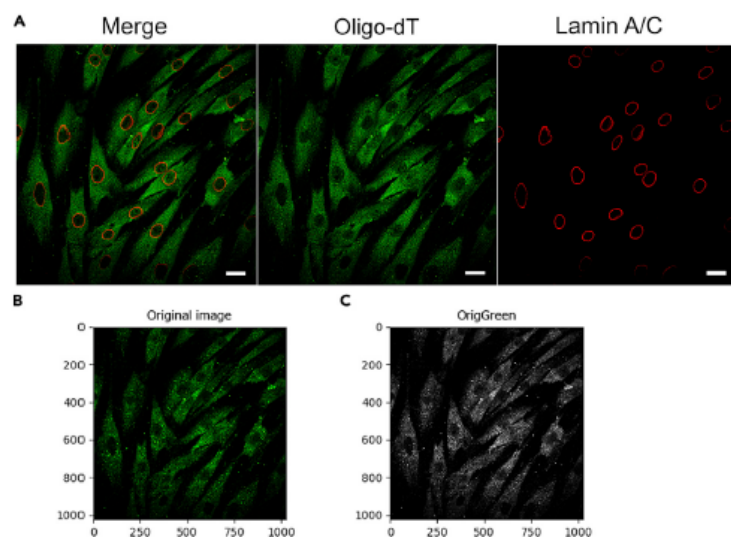


Figure 3. Convert color image to grayscale

(A) Original confocal micrographs of the FISH assay in fibroblast cells. Oligo-dT probe (green) was used to detect mRNAs, and nuclear laminA/C (red) was used to define the nucleus. Scale bar: 20 μ m.

(B) The imported color image of oligo-dT.

(C) The example of grayscale image, which was converted from the color image using CellProfiler software.

9. This step describes the procedure of converting color images to grayscale.

- Insert a new module using the "+" button on the bottom left-hand side of the pipeline. This will open the module search tool.
- Type in "ColorToGray".
- Add the Coloratura module to the pipeline by selecting the module and clicking "+ Add to Pipeline".
- Select the image you would like to input to convert to grayscale. This will be the name you've entered in the NamesAndTypes tab. Depending on your signal, you can select split or combine as your conversion method. For this experiment, channels were split into red and green channels. For overlayed images, channels were combined to convert to grayscale.

Note: Optimal settings will depend on signal strength and crosstalk.

- Select RGB for image type as .tif images are used.
- Depending on the color of the stain, select what channel you would like to convert to gray; Red to gray, green to gray, blue to gray.
- Repeat the previous 6 steps for each image you are using e.g., nucleoplasm signal image, cytoplasmic signal image, and overlayed image (Figure 3).

Settings to define subcellular boundaries

⌚ Timing: 10–30 min

10. This step describes the settings to define subcellular boundaries based on nuclear markers. These settings will allow the software to distinguish the nucleus and the cytoplasm of each cell. The first step is to set the primary object (nuclei) based on nuclear markers, such as nuclear Lamins or DAPI/HST signals.

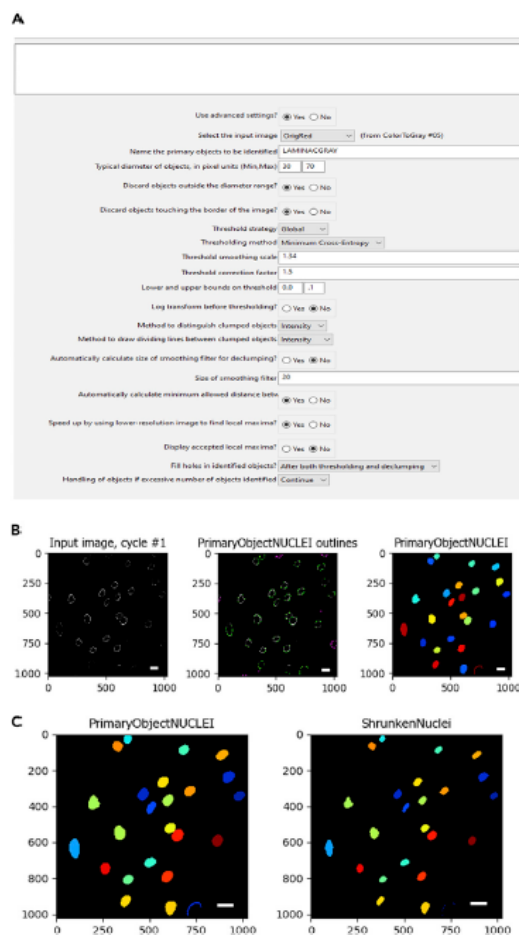


Figure 4. Settings for identification of Primary Object (nuclei)

(A) Identify Primary Objects Module for setting parameters to identify nuclei in confocal micrographs of the FISH assay in fibroblast cells.

(B) Identified nuclei as designated primary objects for analysis. Scale bar: 20 μm .

(C) The primary object nuclei area decreased to avoid overlap with cytoplasmic mRNA signals. Scale bar: 20 μm .

Note: If fluorescence is too sharp compared to the background signal, smoothing may be needed for the identification of edges. This can be accomplished using the “Smooth” module if needed.

Note: Although standard settings may be adequate for identifying nuclei, advanced settings are good for reducing false positives and nuclei that may not be completely in focus. Upon opening advanced settings, select the image you will be used for primary object (nuclei) identification (Figure 4A).

- a. Assign a name to the primary objects identified.



- b. Depending on the nuclei, adjust the maximum and minimum diameter of the primary object to be possessed.

Note: Depending on magnification and cell type, this setting may need adjustment to get the most accurate counts.

- c. To avoid inaccurate signal measurements, select to discard images outside the diameter parameters as well as those touching the edges of the image.
- d. For threshold strategy, select “Global”. This identifies pixels as foreground or background signals.
- e. Select “Manual” as the thresholding method. Depending on the image, the threshold smoothing scale and manual threshold settings may need to be increased or decreased to best properly identify objects.
- f. For the method of distinguishing both clumped objects and dividing lines between clumped objects, select “No”.

Note: Depending on cell type, quality of image, and staining quality, mixing and matching the methods of identifying clumped objects may be needed to produce the most accurate results.

- g. Select “No” for log transforming before thresholding.
- h. Automatically calculating the size of the smoothing filter may be sufficient for discerning different objects. If not, select “No” to automatically calculate the size of the smoothing filter.

Note: If many nuclei are being clumped together and counted as single objects, the value of the smoothing filter may need to be higher to increase the accuracy of counts. As with adjusting the smoothing filter, automatic calculation of the minimum allowed distance between intensity maxima may be sufficient in differentiating objects. If you find incorrect clumping of objects, select “No” for this option.

- i. Lower values for the suppression of local maxima will help in properly splitting nuclei that may still be clumped together as a single object. Select “No” for the options to speed up the processing using a lower resolution image to find local maxima and “No” for the option to display local maxima. If you would like to view local maxima, you can select “Yes”.
- j. Select “After both thresholding and declumping for the option to fill holes in identified objects and select “Continue” for the handling of objects if an excessive number of objects are identified (Figure 4B).
- k. For adjusting nuclei pixel area and getting more accurate signals, open the module search tool as mentioned in the methods of converting images to grayscale. The module is titled “ExpandOrShrinkObjects”. Select the input objects you will be shrinking and name the resulting output objects (e.g., the resulting shrunken nuclei).
- l. Select to shrink the objects by a specified number of pixels.

Note: Depending on the magnification, the number of pixels to shrink can be adjusted, with a higher number being indicative of a greater degree of shrinking of the object (Figure 4C).

11. This step describes the settings for identifying the secondary object (cell body) based on Oligo-dT signals.

Note: Depending on cell type, different images may be used for identifying secondary objects as a lack of signal in the nuclei can produce errors in object identification. The input objects will be the nuclei identified in the previous step. This will be the structure which the secondary objects will be built around and associated with.

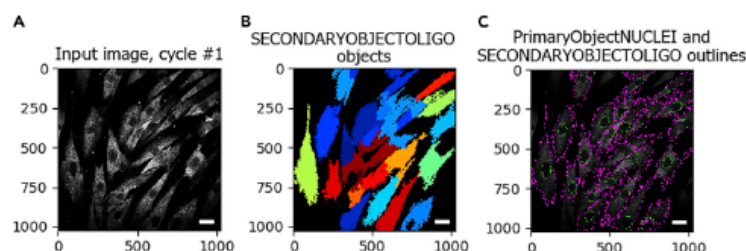


Figure 5. Define secondary object (cell body)

(A) Input image (grayscale).

(B) Secondary object (oligo-dT signal in cell body). Different colors highlight individual cells.

(C) Outlines of the primary (green) and secondary object (purple). Scale bar: 20 μ m.

- a. After assigning a name to the secondary objects, select “Watershed-Gradient” as the method of identifying secondary objects.

Note: The thresholding strategy will be adaptive for the secondary object with the thresholding method being minimum cross-entropy. As with the primary object, settings such as threshold smoothing, and thresholding correction factor can be adjusted to reduce improper clumping of objects.

Note: A value of 0.1 for the threshold smoothing scale and correction factor was found to produce accurate object identification in most cases. Adjusting the lower and upper bounds on thresholds will, after calculating threshold values, allow the program to automatically throw out objects that are outside the calculated threshold. 0.1–1 was found to produce accurate counts in most cases for associated stains.

- b. For the size of an adaptive window, use a multiple of the largest expected object size you estimate will be present. This can vary based on magnification and cell type.
- c. Select “No” for: Log transforming before thresholding and filling holes in identified objects.
- d. Select “No” for discarding secondary objects touching the edges of the image.

Note: Depending on cell type and object areas, you may elect to remove cells that are not 100% in the frame. If done, removing the associated primary objects will also be required to ensure the correct secondary objects are associated with the correct primary objects (Figure 5).

12. This step describes the settings to designate a **tertiary object** (cytoplasm). In this case (the FISH assay of fibroblast cells), the tertiary object will be the cell body minus the nucleus (i.e., the cytoplasmic signal only).

- a. Insert the “IdentifyTertiaryObjects” module.

Note: The larger object will serve as the parent object and will surround the smaller (child) object. In this case, the parent object will be the output generated from the secondary object module and the child object will be the output generated from the expand or shrink module for the primary objects.

- b. Assign a name for the objects identified and select “No” for the option to shrink objects prior to subtraction, as the previous shrink object module has already reduced the primary object’s area (Figure 6).

Note: CellProfiler™ is supposed to correctly associate objects when necessary, but the following steps serve as a means to manually check that the correct nuclei and cell bodies

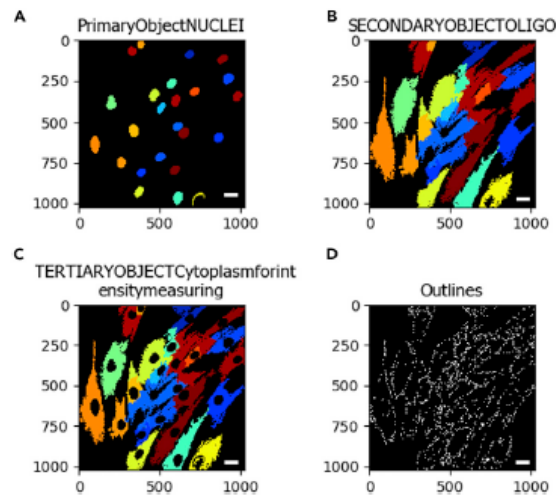


Figure 6. Define tertiary object (cytoplasmic signal)

(A) Primary object (nuclei).
(B) Secondary object (cell body).
(C) Tertiary object (cytoplasm). From A to C, different colors highlight individual objects.
(D) Outlines of the tertiary object. Scale bar: 20 μ m.

are being counted. This helps avoid issues where the nucleus that is in a frame is counted, while the body (secondary object) is excluded due to previously set up parameters.

c. Open the module search tool and load the “DisplayDataOnImage” module into the pipeline.

Note: This module will display the assigned count numbers of each object, allowing you to check that the correct nuclei are assigned to the correct cytoplasm for downstream measurements (Figure 7).

d. Select objects as the measurement and select the input objects you would like to view.

Note: The category will be number, and the measurement will be object number. Select “Yes” for displaying the background image and select the image you would like as the background.

13. This step describes the settings for measuring object intensities and output data.

- Insert the module titled “MeasureObjectIntensity”.
- Select the images you would like to measure.

Note: It is a good idea to choose a variety of images to compare in order to make sure the proper regions/objects are being analyzed.

- Select the object(s) you would like to measure. For the FISH assay, the oligo-dT signal density in the nucleus and the cytoplasm will be measured (Figure 8).
- Insert the “ExportToSpreadsheet” module.

Note: Depending on experiment requirements, you can choose to include or omit certain measurements such as the area and locations objects occupy. This is achieved by selecting “No” for the option to export all data measurements (Figure 9).

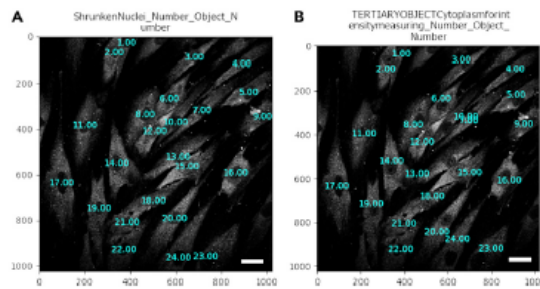


Figure 7. Assigned count numbers of each object

(A and B) Count verification for primary objects (A) and tertiary objects (B). Ensuring that each nuclear signal (primary object) is associated with the correct cytoplasmic signal (tertiary object) for correct measurements using grayscale confocal micrographs. Scale bar: 20 μm .

- e. Based on oligo-dT signal density in the nucleus (Primary Object) and the cytoplasm (Tertiary Object), calculate the ratio of $dT_{(Nuc/Cyt)}$. The higher ratio of $dT_{(Nuc/Cyt)}$ indicates compromised nuclear mRNA export.

Note: Take special note of the names of both images and objects, as the same object may be identified on more than one image.

EXPECTED OUTCOMES

Measure the nuclear mRNA export by FISH assay

We have examined the mRNA distribution in patient-derived motor neurons (MNs) from DYT1 dystonia (a movement disorder) and the healthy controls (Ding et al., 2021). The nuclear mRNA export was measured by the ratio of nuclear to cytoplasmic oligo-dT signals (dT_{Nuc}/dT_{Cyt}). The nuclear dye HST was used to define the nucleus, and the generic neuronal marker MAP2 was used to identify the soma (Figure 10). In healthy control neurons, most mRNAs were localized in the cytoplasm. In contrast, substantial amounts of mRNAs were accumulated inside the nucleus of DYT1 neurons. The specificity of FISH signals was confirmed by the lack of signals when samples were treated with RNase A or when oligo-dA was used as a probe (Figures 10A and 10B). Image analysis and quantification data showed that the ratio dT_{Nuc}/dT_{Cyt} was significantly higher in DYT1 neurons than in control ones (Figure 10D), suggesting that the nuclear mRNA export was impaired in DYT1 neurons.

Select images to measure	☐ DisplayImage	(from DisplayDataOnImage #21)
	☐ DisplayImage	(Duplicate Image Name)
	☐ EnhancedGreen	(from EnhanceOrSuppressFeatures #08)
	☒ FilteredOLIGO	(from Smooth #09)
	☐ LMNAC	(from NamesAndTypes)
Select objects to measure	☐ OLIGO	(from NamesAndTypes)
	☒ OrigGreen	(from ColorToGray #07)
	☐ PrimaryObjectNUCLEI	(from IdentifyPrimaryObjects #10)
	☐ RelateNUCLEICYTOPLASMOBJECTS	(from RelateObjects #19)
	☐ SECONDARYOBJECTOLIGO	(from IdentifySecondaryObjects #16)
	☒ ShrunkenNuclei	(from ExpandOrShrinkObjects #13)
	☒ TERTIARYOBJECTCytoplasmforintensitymeasuring	(from IdentifyTertiaryObjects #17)

Figure 8. Select image and image objects to be analyzed

Options for associating specific images with a specifically identified object that allows measurement of the signal intensity.

Figure 9. Data Export Options for ExportToSpreadsheet module

Module for defining parameters for data output. These parameters, among others, including filtering what data you require, column delimitation, and what values you want such as means, medians, and standard deviations.

Using this protocol, we have also measured the influence of sample sex and neuronal maturation on nuclear mRNA export (Ding et al., 2020). We found that the developmental stages, but not sample sex, affect the mRNA subcellular distribution (Figure 11). These studies demonstrated that the measurement of nuclear mRNA export by FISH assay is reliable and reproducible.

Measure the protein NCT by the dual reporter

The dual reporter system consists of a fused GFP with a NES (GFP-NES) and an RFP fused with NLS (RFP-NLS). In cells with normal NCT activity, GFP and RFP are localized in the cytoplasm and nucleus, respectively; in cells with compromised NCT, this subcellular distribution will be disrupted (Figure 12A). An increased ratio of nuclear to cytoplasmic GFP (GFP_{Nuc}/GFP_{Cyt}) represents compromised protein export, while a higher ratio of cytoplasmic to nuclear RFP (RFP_{Cyt}/RFP_{Nuc}) indicates impaired protein nuclear import. HEK cells were used to validate the expression and the distribution of reporters. As expected, both GFP-NES and RFP-NLS are robustly expressed and almost exclusively distributed in the cytoplasm and the nucleus, respectively (Figure 12B). The exposure to Leptomycin B, a specific nuclear export inhibitor (Jang et al., 2003), caused the signals of GFP-NES gradually accumulated inside the nucleus, but the distribution of RFP-NLS was not affected (Figures 12B and 12C). Using lentiviral delivery of this dual reporter to the reprogrammed human neurons, both GFP-NES and RFP-NLS are highly expressed. Similarly, in neurons that were derived from health fibroblast cells (Sepehrmanesh et al., 2021), GFP and RFP signals are exclusively distributed in the cytoplasm and the nucleus, respectively (Figure 12D).

Using this reporter system, we have examined the protein NCT in reprogrammed DYT1 neurons and healthy controls (Ding et al., 2021). In healthy control neurons, both GFP and RFP were mainly localized in the cytoplasm and nucleus, respectively. In contrast, a substantial amount of GFP accumulated inside the nucleus, and obvious RFP can be noticed in the cytoplasm in DYT1 neurons (Figure 13A). Image analysis and quantification data showed that the ratios of GFP_{Nuc}/GFP_{Cyt} and RFP_{Cyt}/RFP_{Nuc} were significantly higher for DYT1 neurons than for control neurons (Figures 13B and 13C), suggesting that the protein NCT activities are impaired in DYT1 neurons.

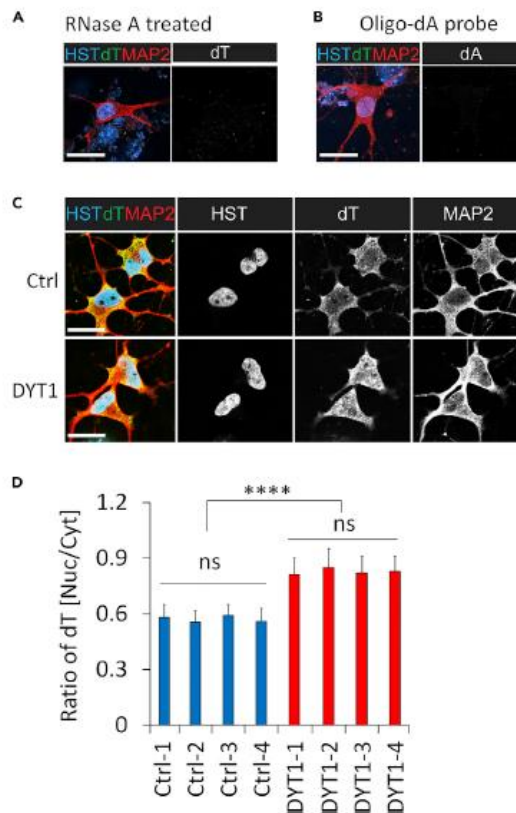


Figure 10. Nuclear mRNA export assay by fluorescence in situ hybridization (FISH) in directly reprogrammed motor neurons (diMNs)

(A) RNase A-treated diMNs as negative controls for fluorescence in situ hybridization (FISH). Cells were analyzed at 6 wpi. MAP2 was used to define the soma and HST (hoechst33342) stained nuclei. Scale bar: 20 μ m.

(B) Oligo-dA probes were used as negative controls for FISH assays at 6 wpi. Scale bar: 20 μ m.

(C) FISH analysis of diMNs with oligo-dT probes at 6 wpi. Scale bar: 20 μ m.

(D) Quantification of oligo-dT signal distribution from four replicates; ns, not significant; **** $p = 3.83E-08$, t-test. Adapted from (Ding et al., 2021).

LIMITATIONS

FISH assay

In this protocol, we combined the FISH assay using DIG-dT probes together with ICC to measure the distribution of total mRNAs. Compared with other methods, in which dT probes are directly conjugated with fluorescent dye (e.g., Quasar 670 etc.) (Aksenova et al., 2020), this protocol used DIG-dT probes. The advantage of DIG-dT probes is that anti-DIG antibody and corresponding secondary antibodies could be employed to amplify the signal. At the same time, different combinations of antibodies could be used for detecting transcripts and other protein markers by ICC, practically important if you need to distinguish different cell types that are co-cultured on a single coverslip. One limitation of the FISH assay in this protocol is that antibody staining could give rise to non-specific signals. We usually set up two negative controls to verify the specificity of FISH results (Figures 10B and 10C). If new antibodies will be used in the assay, you also need to set antibody controls, including both primary and secondary antibodies. This protocol only described the

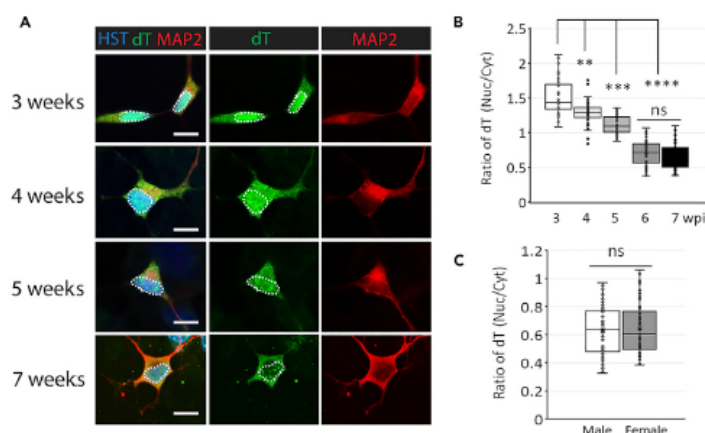


Figure 11. Influence of developmental stages and sex on mRNA subcellular distribution in reprogrammed human neurons

(A) A time course analysis of mRNA distribution by FISH in diMNs from 3 to 7 wpi. MAP2 was used to define the soma and HST (hoechst33342) stained nuclei. Nuclei are highlighted in circular dotted lines. Scale bars: 10 μ m.

(B) Quantification of oligo-dT signal distribution in diMNs at indicated time points. Ratios of the nuclear to the cytoplasmic dT signal density (Nuc/Cyt) were used to show the mRNA distribution. n (neurons) = 123 for 3 wpi, n = 112 for 4 wpi, n = 115 for 5 wpi, n = 102 for 6 wpi, n = 96 for 7 wpi from triplicates. ns, not significant; **p<0.01; ***p<0.001; ****p<0.0001.

(C) Quantification of oligo-dT signal distribution in male- and female- diMNs at 6 wpi. n (neurons) = 322 for male and n = 316 for female from triplicates. ns, not significant. Adapted from (Ding et al., 2020).

measurement of mRNAs using dT probes. If you need to measure some gene-specific mRNAs or other types of RNAs, sequence-specific probes need to be designed and synthesized.

Dual report assay

Protein nuclear transport has been used more frequently to evaluate the NCT activities. There are several strategies to evaluate the protein movement across the nuclear membrane. Based on immunostaining, the subcellular localization of specific endogenous proteins could be examined, such as the NFAT (nuclear factor of activated T cells) transcription factors that require the changes of the subcellular localization to fulfill their transcriptional regulations (Ding et al., 2018; Muramatsu et al., 2021). In this protocol, the protein transport activities will be measured by monitoring the kinetics of ectopically expressed reporters, GFP-NES and RFP-NLS. Compared with other alternative methods, there are several advantages of this protocol. First, the signals of GFP-NES and RFP-NLS could be simultaneously examined to evaluate both export and import activities in single cells. Second, GFP and RFP signals can be visualized directly under a fluorescent microscope and samples do not need to be stained with antibodies. Third, cultured cells expressing reporters can be visualized in living condition, and do not need to be fixed and/or permeabilized, making it possible to reveal the protein transport activities under a more physiological condition.

One limitation of the protein transport assay using this reporter system is that lentivirus needs to be prepared for the transduction of postmitotic cells such as primary cells or reprogrammed neurons. The quality of lentivirus will significantly influence the transduction efficiency and the expression levels of reporters (Ding and Kilpatrick, 2013). The proper signal density of reporters is important to give rise to reliable results in the following quantification assay. The growth condition of different cell lines may also affect the transduction efficiency. Another limitation is that the protein nuclear transport activities measured by this dual report only reveal the overall transport activities for proteins contain typical NLS or NES signal peptide. For certain protein cargos that use different

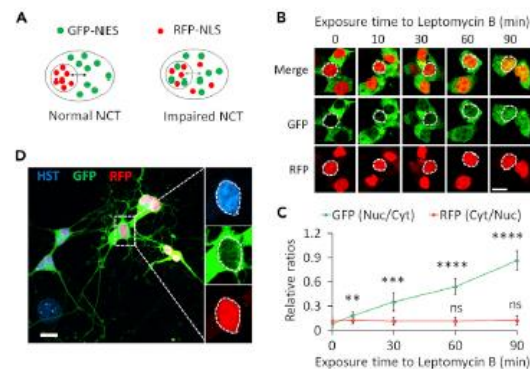


Figure 12. Protein nucleocytoplasmic transport (NCT) measurement with a dual reporter

(A) A schematic shows a dual reporter system for measuring protein NCT. GFP: green fluorescent protein; NES: nuclear export sequence; RFP: red fluorescent protein; NLS: nuclear localization sequence.
(B) Representative confocal micrographs of HEK cells expressing the dual reporter with and without Leptomycin B (50 nM) treatment. Circular dotted lines highlight the nuclei. Scale bar: 10 μ m.
(C) Ratios of nuclear to cytoplasmic GFP-NES (Nuc/Cyt) and cytoplasmic to nuclear RFP-NLS (Cyt/Nuc) in HEK cells shown in B. n (cells) > 100 for each time points from triplicates. Compared to control (0 min), ns, not significant; **p < 0.01, ***p < 0.001; ****p < 0.0001.
(D) A representative confocal micrograph of reprogrammed human neurons (health) expressing the dual reporter at 6 wpi. The highlighted area was also shown at large magnification and the nucleus is highlighted by a circular dotted line based on HST signal. Scale bar: 10 μ m. Adapted from (Ding et al., 2020).

signaling peptides or different signaling pathways, their transport and subcellular distribution need to be measured specifically, such as by antibody-based ICC assay.

Image analysis

Confocal images of FISH assay or reporter assay could be analyzed using software such as ImageJ or CellProfiler™ either manually or automatically with specific settings based on the purpose. Usually, if you have low number of samples, quantify images manually will work. If you need to quantify a large number of cells, you may need to set up a method using some software for automagical image analysis. In this protocol, we used CellProfiler to quantify signal density and subcellular distribution. CellProfiler is a free, open-source software that allows to automatically quantify images. Compared with other software, CellProfiler is easy to use and requires little programming knowledge. It offers pipeline saving option including modules and settings. This feature enables CellProfiler to generate reproducible data. At a single cell level, the subcellular distributions of dT signal or fluorescent proteins are measured, and the ratios of cargos' subcellular distributions are calculated to evaluate the NCT activities. For each cell, as large an area as possible to be measured within the nucleus or cytoplasm. To avoid potential bias, we recommend an unbiased approach for data collection. For example, the person who will analyze the images will be completely blinded to the sample information.

TROUBLESHOOTING

Problem 1

Weak oligo-dT signal in the FISH assay.

The oligo-dT signal is weak in the FISH assay (steps 1–3).

Potential solution

The weak FISH signal could be caused by several reasons. 1) The degradation of mRNAs in samples. To prevent potential RNA degradation, it is important to prepare all related solutions using

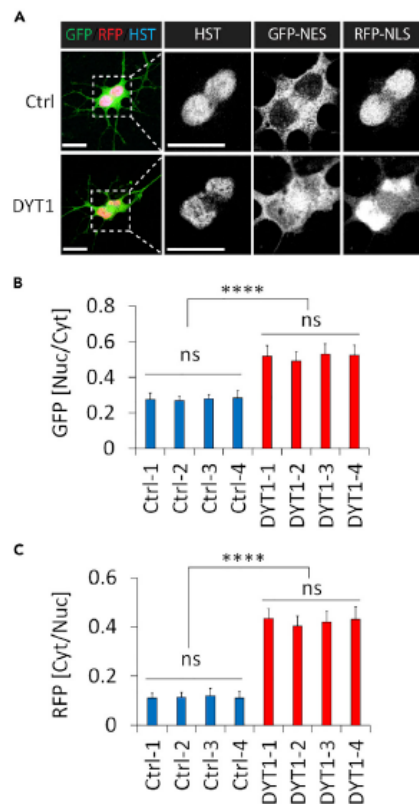


Figure 13. Protein nucleocytoplasmic transport assay in reprogrammed human neurons

(A) Representative confocal images of the reporter distribution in healthy and DYT1 diMNns at 6 wpi. HST stained nuclei. Scale bar: 20 μ m.

(B) Subcellular distribution of GFP-NES from four replicates; ns, not significant; **** $p < 0.0001$.

(C) Subcellular distribution of RFP-NLS from four replicates; ns, not significant; **** $p < 0.0001$. Adapted from (Ding et al., 2021).

nuclease-free water. If samples cannot be processed immediately after fixation, you may store them at -20°C in 70% ethanol for a few weeks without an obvious decrease of oligo-dT signals, but the signal quality of some protein markers could be diminished. 2) Low concentration or poor quality of oligo-dT probes. Make fresh probes with proper concentration. 3) Antibodies. Make sure the antibodies used are of good quality.

Problem 2

High background of FISH signal in negative controls.

Substantial oligo-dT signals can be noticed in negative control samples in the FISH assay (steps 1–3).

Potential solution

To validate the specificity of oligo-dT signals, two negative controls are recommended. One negative control is the sample control, which is treated with RNase A to degraded RNAs. The second negative control is the probe control, in which samples are hybridized with oligo-dA probes that

will not bind to mRNAs. If the RNase A treated samples show high background, the RNase A reagent and the digestion conditions need to be validated. The high background of oligo-dA probe control could be caused by the cross contamination with oligo-dT probes during hybridization. To systematically decrease the background signals, more stringent wash conditions need to be considered by adding more times of wash and/or washing longer each time. If necessary, antibody controls could be added to validate the specificity of new antibodies. These negative controls are processed simultaneously with other samples. The specificity of oligo-dT signals could be verified if negative controls show no or very little signals under the same confocal settings (Figures 10B and 10C).

Problem 3

Low expression of the dual reporter in transduced cells.

In transduced cells, the expression level of the dual reporter is low (step 7).

Potential solution

The low expression of the dual reporter in transduced cells is usually caused by the poor quality of lentivirus. To prepare high-quality lentivirus and validate the titer, please refer to our previous reports (Akte et al., 2022; Ding and Kilpatrick, 2013; Sepehrmanesh et al., 2021).

Problem 4

More cells died after transduction.

Large quantities of transduced cells died (more frequently occurs in neurons) (steps 6 and 7).

Potential solution

More cells dying is usually caused by the transduction of a very high dosage of the virus. To achieve a reasonable infection efficiency, the dosage of the virus should be optimized to transduce different cell types. Too much virus may cause more cell death. The transduction efficiency and the cell survival should be well balanced. Generally, about 60% transduction efficiency is good enough to examine the protein transport in most samples. We usually employ 5 MOI (multiplicity of infection) to infect fibroblast cells, and around 2 MOI to transduce primary or iPSC-derived neural progenitor cells. For transduction of post-mitotic and differentiated neurons, we suggest using 3 or 4 MOIs.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Baojin Ding (baojin.ding@lsuhs.edu).

Materials availability

The majority of materials required in this protocol are commercially available.

Data and code availability

This study did not generate/analyze any datasets or code that were submitted to any repositories.

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AUTHOR CONTRIBUTIONS

Conceptualization, B.D.; Methodology, B.D., H.C., and C.C.; Investigation, B.D., H.C., C.C., M.S., M.A., and M.A.H.; Writing, B.D., M.S., H.C., and C.C.; Funding Acquisition and Supervision, B.D.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Appendix E- Abstract-Oral presentation 1

April 27, 2023. *Patient-specific neurons are key to new discoveries in neurological diseases.* The Global Experts meet on Neurology, Neuroscience and Brain Disorders. April 27-29, 2023. Paris, France. (Invited Speaker, In Person)



Global Experts Meet on

Neurology, Neuroscience and Brain Disorder

Venue: Paris, France
Dates: April 27-29, 2023.

Patient-specific neurons are key to new discoveries in neurological diseases



Baojin Ding

Department of Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center, Shreveport, LA USA

Abstract:

The limited access to human neurons greatly impedes the progress of research in neurological diseases. Although animal models provide insights into disease mechanisms, significant species-dependent differences exist, and animal models only mirror the limited aspects of the pathophysiology of human diseases. It is believed that these species-dependent differences caused the high failure rate in clinical trials that have been derived from successful results in animal models. Excitingly, reprogramming of human neurons from adult fibroblasts overcomes this limitation and provides an unprecedented approach in deciphering the molecular pathogenesis underlying neurological diseases. Recently, we have reported two techniques for the generation of patient-specific neurons. One is the direct conversion from patient fibroblasts using lentiviral delivery of transcription factors [1], and the second method is induced pluripotent stem cells (iPSCs)-based induction and differentiation [2, 3]. Using different combinations of reprogramming factors, different neuronal subtypes could be generated, such as generic neurons (iGNs), motor neurons (iMNs), and cholinergic interneurons (iChINs). We modelled the movement disorder DYT1 dystonia using patient-specific neurons and recapitulated the disease-dependent cellular deficits, including deformed nucleus, impaired neurodevelopment, disrupted nucleocytoplasmic transport, and the unexpected finding of nuclear Lamin B1 mislocalization [4, 5]. This study provided the novel insights into the pathogenesis of dystonia and revealed the high value of patient-derived neurons in modelling neurological diseases.

Appendix F- Abstract- Oral presentation 2

June 15, 2022. *Modeling Neurological Diseases Using Patient-derived Neurons*. The 5th Edition of International Conference on Neurology and Neurological Diseases. June 15-16, 2022. (Invited Speaker. Virtual)

5th Edition of International Conference on **Neurology And Neurological Disorders**

June 15-16, 2022 | Virtual Event

Modeling Neurological Diseases Using Patient-derived Neurons

Baojin Ding

Department of Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center in Shreveport, Shreveport, LA USA.

Correspondence: baojin.ding@lsuhs.edu

Abstract:

The limited access to human neurons greatly impedes the progress of research in neurological diseases. Although animal models provide insights into disease mechanisms, significant species-dependent differences exist, and animal models only mirror the limited aspects of the pathophysiology of human diseases. It is believed that these species-dependent differences caused the high failure rate in clinical trials that have been derived from successful results in animal models. Excitingly, reprogramming of human neurons from adult fibroblasts overcomes this limitation and provides an unprecedented approach in deciphering the molecular pathogenesis underlying neurological diseases. Recently, we have reported two techniques for the generation of patient-specific neurons. One is the direct conversion from patient fibroblasts using lentiviral delivery of transcription factors, and the second method is induced pluripotent stem cells (iPSCs)-based induction and differentiation. Using different combinations of reprogramming factors, different neuronal subtypes could be generated, such as generic neurons (iGNs), motor neurons (iMNs), and cholinergic interneurons (iChINs). We modeled the movement disorder DYT1 dystonia using patient-specific neurons and recapitulated the disease-dependent cellular deficits, including deformed nucleus, impaired neurodevelopment, disrupted nucleocytoplasmic transport, and the unexpected finding of nuclear Lamin B1 mislocalization. This study provided the novel insights into the pathogenesis of dystonia and revealed the high value of patient-derived neurons in modeling neurological diseases.

What will audience learn from your presentation?

1. Two novel techniques for the generation of patient-specific neurons.
2. The generation of different neural subtypes via different reprogramming factors.
3. The differences and similarities of directly converted neurons and iPSC-derived neurons in modeling neurological diseases.
4. The novel insights into the pathogenesis of DYT1 dystonia in patient-specific neurons.

Note: The techniques for the generation of patient-derived neurons and the related research resources can be found in our recent publications (PMID: 35300000, 34746870, 34536661, 34380890, 33468570, 33510083, 32783653, 32317929).

Biography of presenting author (should not exceed 100 words)

Dr. Baojin Ding received his bachelor's degree in Medicine (MD equivalent) and master's degree in Clinical Laboratory in China. After he received PhD in Biochemistry and Molecular Biology from Louisiana State University (LSU), he did postdoctoral training in Neuroscience at the UMass Medical School and worked as a research faculty in the UT Southwestern Medical Center. In 2018, Dr. Ding obtained an Assistant Professor (tenure-track) at the UL Lafayette and then relocated to LSU Health Shreveport. The research in Dr. Ding's laboratory is focusing on Molecular and Cellular Neuroscience and Neurological Diseases, and currently funded by NIH and DoD.

Details of presenting author to be mentioned in certificate:

Name: Baojin Ding

Affiliation: Department of Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center in Shreveport

Country: United State of America

Other Details:

Presentation Category: Oral Presentation

Session Name:

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Contact Number: +1 (318) 675-5178

Twitter/Facebook/LinkedIn: www.DingLabBiomed.com

Suggestion of speakers to be invited: Names and email address of your colleagues or friends interested to attend.

Recent Photograph: (High Resolution)



If you feel difficulty in inserting high resolution image in word document, please send it in email at neuro@magnusconference.com

Appendix G- Abstract-Poster presentation

December 4, 2022. **Ding B.** *Impaired Nucleocytoplasmic Transport in Neurological Diseases*. American Society for Cell Biology (ASCB) Annual Meeting. Washington DC. December 3-7, 2022. (In person)



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Activity: Abstract

Current Date/Time: 8/4/2022 8:33:20 AM

Impaired Nucleocytoplasmic Transport in Neurological Diseases

Author Block: B. Ding;

Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center, Shreveport, LA.

Abstract:

In eukaryotic cells, transcription and translation processes are physically separated by the nuclear envelope (NE). Newly transcribed mRNAs must be exported to the cytoplasm for protein synthesis, while some proteins require to be imported into the nucleus to fulfill their nuclear functions. This nucleocytoplasmic transport (NCT) across the NE is tightly regulated and is critical for maintaining cellular homeostasis. Its dysregulation leads to aging and many neurological diseases, including amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), and Alzheimer's disease (AD). To decipher the pathophysiological mechanisms underlying NCT-impaired neurological diseases, the examination of NCT activities at the single cell level is critical. Recently, we reported the techniques for measuring the nuclear transport of both mRNA and protein cargos. *Fluorescent In Situ Hybridization* (FISH) coupled with oligo-dT probes were used to measure the distribution of Poly(A) RNAs (mRNA). A dual reporter (GFP-NES and RFP-NLS) was used to examine the protein nuclear transport. These approaches together with imaging analysis enable us to systematically quantify the NCT activities in cultured neurons. We modeled movement disorder dystonia using patient-derived motor neurons (MNs), which have been generated via direct conversion from patient fibroblasts and the differentiation of induced pluripotent stem cells (iPSCs). We first reported the disease-dependent cellular deficits of dystonia in patient-derived neurons, including deformed nucleus, mislocalized proteins, and impaired nucleocytoplasmic transport, providing another example of the impairment of NCT in neurological diseases. Our study also demonstrates the high value of patient-derived neurons in modeling neurological diseases.

Author Disclosure Information:

B. Ding: PI., This work was supported by NIH grant (NIH/NINDS NS112910 to B.D.) and Department of Defense (DoD) Peer Reviewed Medical Research Program (PRMRP) Discovery Award (W81XWH2010186 to B.D.).

Scientific Track(s) (Complete): Cellular Dynamics: Compartmentalization, Trafficking, Cytoskeleton, Division, and Migration ; Cells in Distress and Disease: Cancer, Aging, Infection, Stress, Chemical Biology, and Therapeutics

Presenting Author Information (Complete):

Please indicate the presenting author career status: Assistant Professor or Equivalent

Male : True

Does the presenting author belong to one of these historically excluded communities?: NO

Asian : True

: No

Presentation Selections (Complete):

Please indicate the publishing status of your research submitted in your abstract: Some of this research has been published

Minisymposia Talk : True

Poster : True

Topic 1: +9a Nucleocytoplasmic transport

Topic 2: +18b Dynamics of proteins and organelles in neurons

Subgroup Topic: Membrane Trafficking and Proteostasis in Neurons: Neurodegeneration, Autophagic, and Endolysosomal Pathways

Symposia Topic: Cellular and Organelle Movement during Health and Disease (Sunday)

If you are selected for a talk, will you also present a poster?: Yes

Does your abstract contain new technology?: Yes

Payment (Complete): Your credit card order has been processed on Thursday 28 July 2022 at 3:25 PM.

Status: Complete

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Appendix H-the PI's updated CV

CURRICULUM VITAE
Baojin Ding M.D., PH.D.



TITLE AND CONTACT INFORMATION

Assistant Professor
Department of Biochemistry and Molecular Biology
LSU Health Shreveport
1501 Kings Highway, Shreveport, LA 71130
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RM. F7-33, 49 (Lab); F7-23 (Office)
E-mail: baojin.ding@lsuhs.edu;
Tel: (318) 675-5178 (Office), (318) 675-5179 (Lab)

EDUCATION

- 2010** Ph.D. in Biochemistry and Molecular Biology of Veterinary Medical Sciences,
Louisiana State University, School of Veterinary Medicine, Baton Rouge, LA
- 2004** M.S. of Medicine in Clinical Laboratory
Wenzhou Medical College, Wenzhou, Zhejiang, P. R. China
- 2001** bachelor's degree of Medicine (M.D. equivalent)
Medical College of Qingdao University, Qingdao, Shandong, P. R. China

PROFESSIONAL EXPERIENCE

2022- Present: Assistant Professor (Tenure track)

Department of Biochemistry and Molecular Biology, LSU Health Shreveport, 1501 Kings Highway, Shreveport, LA 71130
Research area: Biochemistry and Molecular Biology, Neuroscience and Neurological Diseases.

2018- 2021: Assistant Professor (Tenure track)

Department of Biology, University of Louisiana at Lafayette, Lafayette, LA 70503
Research area: Cell Biology, Neuroscience and Neurological Diseases.

2016- 2018: Assistant instructor

Department of Molecular Biology, Center for Regenerative Science and Medicine,
UT Southwestern Medical Center, Dallas, TX
Research interests: Nucleocytoplasmic transport in neural development and neurological disorders.

2013-2016: Postdoctoral Fellow

Department of Neurobiology, University of Massachusetts Chan Medical School, Worcester, MA
Research topic: Regulatory mechanisms of nuclear mRNA export.

2010- 2013: Postdoctoral Fellow

Departments of Microbiology and Physiological Systems (MaPS), University of Massachusetts Chan Medical School, Worcester, MA
Research topic: Molecular mechanisms of gene expression in neurodevelopment and its linkage to neurological disorders.

2005- 2010: Graduate Research Assistant

Department of Comparative Biomedical Science, School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA
Research topic: The Molecular Mechanisms of Transcription Coupled DNA Repair in Eukaryotic cells.
(Ph.D. Dissertation: http://etd.lsu.edu/docs/available/etd-04072010-012548/unrestricted/Ding_diss.pdf)

2004- 2005: Research Associate

Department of Biological Sciences, Louisiana State University, Baton Rouge, LA
Research topic: The Biogenesis and Functions of Iron-Sulfur Cluster proteins.

2002- 2004: Research Assistant

The Institute of Cellular and Molecular Medicine, Wenzhou Medical University, Wenzhou, Zhejiang, P. R. China

Research topic: Isolation, Purification and Screening of Effective Components from Natural Products.

2000- 2001: Intern Doctor

Affiliated Hospital of Medical College of Qingdao University, Qingdao, P. R. China

HONORS AND AWARDS

2020-2023	South Louisiana Mid-Winter Fair Association/BORSF Professorship
2020	DoD Peer Reviewed Medical Research Program (PRMRP) Discovery Award
2017	Alzheimer's Disease Research Award from NIH Alzheimer's Disease Center and Friends of the Alzheimer's Disease Center.
2013	American Association of Anatomists (AAA) Travel Award
2014	American Association of Anatomists (AAA) Travel Award
2010	American Society for Microbiology Postdoctoral Research Fellowship Program.
2002	Guang Hua Scholarship
2003	Guang Hua Scholarship
2000	Undergraduate Scholarship
1999	Excellent Student Award
1998	Undergraduate Scholarship
1997	Excellent Student Award
1997	Undergraduate Scholarship

RESEARCH SUPPORT**Current: (As of September 24, 2023)**

- Title:** Modeling DYT1 Dystonia in Patient-derived Neurons
Agency: NIH/NINDS
Type: Research Grant
Grant Number: R56NS133252
Role: Principal Investigator
Dates: 09/01/2023 - 08/31/2024
Total amount:
Overall goal: To develop novel cellular systems for dystonia research and biochemically identify dysregulated factors (genes and proteins) and functional alterations using patient-derived neurons.
- Title:** Determining the Pathogenesis of Dystonia in Reprogrammed Human Neurons
Agency: DoD Peer Reviewed Medical Research Program (PRMRP) Discovery Award
Type: Research Grant
Grant Number: W81XWH2010186
Role: Principal Investigator
Dates: 03/15/2020 - 03/14/2024 (approved for NCE)
Total amount:
Overall goal: To determine the pathogenesis of adulthood onset dystonia via directly reprogramming human neurons from patient fibroblasts.

3. **Title:** Determining the Pathogenesis of DYT1 Dystonia in Reprogrammed Human Neurons
Agency: NIH/NINDS
Type: Research Grant
Grant Number: R21 NS112910
Role: Principal Investigator
Dates: 04/01/2020 - 09/30/2023
Total amount:
Overall goal: To determine the pathogenesis of childhood onset DYT1 dystonia in patient-specific neurons that are generated by direct conversion and iPSC-based induction and differentiation.
4. **Title:** Biochemically identify dysregulated factors in DYT1 dystonia via iPSC-derived neurons
Agency: Center for Brain Health, LSU Health Shreveport
Type: Research Grant
Grant Number: CBH Grant-In-Aid Spring 2022
Role: Principal Investigator
Dates: 07/01/2022 - 12/30/2023
Total amount:
Overall goal: This intramural grant aims to help generate more preliminary data for applying extramural awards.
5. **Title:** Seed Package
Agency: Department of Biochemistry and Molecular Biology and LSU Health Shreveport
Type: Startup grant
Grant Number: 5101501078
Role: Principal Investigator
Dates: 2022 - 2025
Total amount:
Overall goal: A seed package is to assist PI in establishing the laboratory, purchasing equipment and supplies essential to the research, hiring research personnel, and furnishing office.

Completed:

1. Undergraduate Research Apprenticeship Program (UGRAP), 2023 Summer Pipeline.
2. Jumpstart Summer Enrichment program (JSEP), 2023 Summer Pipeline.
3. **Title:** South Louisiana Mid-Winter Fair Association/BORSF Professorship
Agency: University of Louisiana at Lafayette
Type: Professorship
Grant Number: 08178
Role: Principal Investigator
Dates: 2020 - 2023
Total amount:
4. Jumpstart Summer Enrichment (JSEP) program, 2022 Summer Pipeline.
5. **Title:** Lahaye Faculty Development Grant
Agency: University of Louisiana at Lafayette
Type: Startup grant
Grant Number:
Role: Principal Investigator
Dates: 2019 - 2020
Total amount:
Overall goal: This fund is intended to support undergraduate researchers working on biomedical studies.

6. **Title:** Enhancement of Biology Research and Teaching through Personal Flow Cytometry
Agency: Louisiana Board of Regents
Type: Grant
Grant Number:
Role: Co-Principal Investigator (PI: B. Stauffer)
Dates: 06/01/2020-05/31/2021,
Total amount:
Overall goal: To improve the educational and research infrastructure in the department of Biology in UL Lafayette.
7. **Title:** Nucleocytoplasmic Transport Defect in to Alzheimer's disease
Agency: NIH/NIA
Type: Research Grant
Grant Number: NIH/NIA P30-12300-21
Role: Co-Principal Investigator
Dates: 2017 -2018
Total amount:
Overall goal: To understand how nucleocytoplasmic transport defect contributes to Alzheimer's disease (AD) by using mouse primary neurons, mammalian cell lines and directly reprogrammed neurons (diNs) from fibroblasts of AD patients.

Pending:

1. **Title:** Molecular mechanisms of Torsin AAA+ ATPase in the regulation of nucleocytoplasmic transport
Agency: NSF
Type: Research Grant
Grant Number:
Role: Principal Investigator
Dates: 10/01/2023 - 09/30/2027
Total amount:
Overall goal: To determine the molecular mechanisms of Torsin ATPase in the regulation of nucleocytoplasmic transport

PUBLICATIONS (*corresponding author)

<https://www.ncbi.nlm.nih.gov/myncbi/baojin.ding.1/bibliography/public/>

1. Masuma Akter, Haochen Cui and **Baojin Ding***. Overexpression of RANBP17 ameliorates the impaired nucleocytoplasmic transport in DYT1 patient-derived neurons. **J Neuroscience**. (Under review)
2. **Baojin Ding***, Masood Sepehrimanesh, Jacob Stagra. Dysregulated nuclear Lamin B1 disrupts 14-3-3 proteins in DYT1 dystonia motor neurons. **iScience**. (Under revision)
3. Akter M, Cui H, Hosain A., and **Ding B***. (2023). Generation of two induced pluripotent stem cell lines with heterozygous and homozygous amyotrophic lateral sclerosis mutation P525L (c.1574C>T) in FUS gene. **Stem Cell Research**. 2023 Apr 24; 69:103103. <https://doi.org/10.1016/j.scr.2023.103103>
4. Akter M, Cui H, Hosain A., and **Ding B***. (2023). Generation of two induced pluripotent stem cell lines with heterozygous and homozygous amyotrophic lateral sclerosis mutation R521G (c.1561C>G) in FUS gene. **Stem Cell Research**. 2023 Mar 21; 69:103078. <https://doi.org/10.1016/j.scr.2023.103078>
5. Masuma Akter, **Baojin Ding***. Modeling Movement Disorders via Generation of hiPSC-Derived Motor Neurons. In: Sahar Esfandyari, editor. **Prime Archives in Molecular Biology: 2nd Edition**. Hyderabad, India: Vide Leaf. 2023. (Book Chapter)
6. Akter M and **Ding B***. (2022). Modeling Movement Disorders via Generation of hiPSC-Derived Motor Neurons. **Cells**. 2022 Nov 27;11(23):3796. <https://doi.org/10.3390/cells11233796>

7. Cui H, Sepehrimanesh M, Coutee C, Akter M, Hosain A and **Ding B***. (2022). Protocol to image and quantify nucleocytoplasmic transport in cultured cells using fluorescent in situ hybridization and a dual reporter system. **STAR Protoc.** 2022 Dec 16; 3(4):101813.
<https://doi.org/10.1016/j.xpro.2022.101813>
8. Akter M, Cui H, Chen Y-H and **Ding B***. (2022). Generation of gene-corrected isogenic control cell lines from a DYT1 dystonia patient iPSC line carrying a heterozygous GAG mutation in TOR1A gene. **Stem Cell Research.** 62:July 2022, 102807. <https://doi.org/10.1016/j.scr.2022.102807>
9. Akter M, Cui H, Sepehrimanesh M., Hosain A. and **Ding B***. (2022). Generation of highly pure motor neurons from human induced pluripotent stem cells. **STAR Protoc.** 2022 Mar 10; 3(1):101223. (Invited protocol. Cell Press)
<https://doi.org/10.1016/j.xpro.2022.101223>
10. **Ding B***. (2022). Novel insights into the pathogenesis of DYT1 dystonia from induced patient-derived neurons. **Neural Regen Res** 17(3):561-562. (Invited Perspective)
11. Sepehrimanesh M., Akter M. and **Ding B***. (2021). Direct conversion of adult fibroblasts into motor neurons. **STAR Protoc.** 2 (4) 17 December 2021, 100917 (Invited protocol, Cell Press).
<https://doi.org/10.1016/j.xpro.2021.100917>
12. Akter M, Cui H, Chen Y-H and **Ding B***. (2021). Generation of two induced pluripotent stem cell lines with heterozygous and homozygous GAG deletion in TOR1A gene from a healthy hiPSC line. **Stem Cell Research.** 56 (2021)102536.
<https://doi.org/10.1016/j.scr.2021.102536>
13. **Baojin Ding*** and Masood Sepehrimanesh. Nucleocytoplasmic Transport: Regulatory Mechanisms and the Implications in Neurodegeneration. In: Letizia Giampietro, editor. **Prime Archives in Molecular Sciences: 2nd Edition.** Hyderabad, India: Vide Leaf. 2021. (Book Chapter)
14. **Ding B*** and Sepehrimanesh M. (2021). Nucleocytoplasmic transport: regulatory mechanisms and the implications in neurodegeneration. **Int. J. Mol. Sci.** 22 (8): 4165.
<https://doi.org/10.3390/ijms22084165>
15. **Ding B***, Tang Y, Ma S, Akter M, Liu ML, Zang T, Zhang CL. (2021). Disease modeling with human neurons reveals LMNB1 dysregulation underlying DYT1 dystonia. **J Neurosci.** 41 (9): 2024-2038.
(Featured Article, <https://www.jneurosci.org/content/41/9/1846>
Selected as **JNeurosci's Annual Spotlight 2021. JNeurosci's Annual Spotlight features articles that received the highest marks for both methodological merit and significance.**)
16. **Ding B***. (2021). Generation of patient-specific motor neurons in modeling movement diseases. **Neural Regen Res** 16(9):1799-1800.
17. **Baojin Ding***, Yu Tang, Shuaipeng Ma, Meng-Lu Liu, Tong Zang, Masuma Akter and Chun-Li Zhang (2020). Disease modeling with human neurons reveals LMNB1 dysregulation underlying DYT1 dystonia. **bioRxiv.** doi: <https://doi.org/10.1101/2020.08.11.246371>
18. Sepehrimanesh M, and **Ding B*** (2020). Generation and Optimization of Highly Pure Motor Neurons from Human Induced Pluripotent Stem Cells via Lentiviral Delivery of Transcription Factors. **Am J Physiol Cell Physiol.** 319: C771–C780.
19. **Ding B,*** Akter M, and Zhang C-L. (2020). Differential Influence of Sample Sex and Neuronal Maturation on mRNA and Protein Transport in Induced Human Neurons. **Front Mol Neurosci.** 2020 Apr 3; 13: 46.
20. Selvam K, **Ding B**, Sharma R and Li S. (2019). Evidence that moderate eviction of Spt5 and promotion of error-free transcriptional bypass by Rad26 facilitates transcription coupled repair. **J Mol Biol.** 2019 Feb 18. doi: 10.1016.
21. **Ding B**, Dobner PR, Mullikin-Kilpatrick D, Wang W, Zhu H, Chow CW, Gronostajski RM and Kilpatrick DL. (2018). BDNF Activates an NFI-Dependent Neurodevelopmental Timing Program By Sequestering NFATc4. **Mol Biol Cell.** 2018 Apr 15; 29(8):975-987.

22. **Ding B.**, Mirza A.M., Alshley J. Budnik V. and Munson M. (2017). Nuclear Export Through Nuclear Envelope Remodeling in *Saccharomyces cerevisiae*. **bioRxiv** 224055; doi: <https://doi.org/10.1101/224055>
23. Leto K, Arancillo M, Becker EB, Buffo A, Chiang C, **Ding B**, Dobyns WB, Dusart I, Haldipur P, Hatten ME, Hoshino M, Joyner AL, Kano M, Kilpatrick DL, Koibuchi N, Marino S, Martinez S, Millen KJ, Millner TO, Miyata T, Parmigiani E, Schilling K, Sekerková G, Sillitoe RV, Sotelo C, Uesaka N, Wefers A, Wingate RJ, Hawkes R. (2016). Consensus Paper: Cerebellar Development. **Cerebellum**. Dec;15(6): 789-828.
24. Li Y, Hassinger L, Thomson T, **Ding B**, Ashley J, Hassinger W and Budnik V. (2016). Lamin Mutations Accelerate Aging via Defective Export of Mitochondrial mRNAs through Nuclear Envelope Budding. **Curr Biol**. 2016 Aug 8;26(15):2052-9.
25. **Ding B**, Cave HW, Dobner PR, Kilpatrick DM, Bartsokis M, Zhu H, Chow CW, Gronostajski RM and Kilpatrick DL. (2016) Reciprocal Auto-Regulation by NFI Occupancy and ETV1 Promotes the Developmental Expression of Dendrite-Synapse Genes in Cerebellar Granule Neurons. **Mol Biol Cell**. 2016 May 1;27(9):1488-99.
26. **Ding B***. (2015) How does a 1.5-Fold Increase in Gene Dosage in Chromosome 21 Cause the Pleiotropic Phenotypes in Down Syndrome? **J Down Syndr Chr Abnorm** 1: 1: e101. doi:10.4172/jdsca.1000e101 (Editorial)
27. **Ding B***. (2015) How to Assist Parents of Children with Autism Spectrum Disorders in Rural Area? **J Neurosci Rural Pract**. 6 (4), 465-6 (Editorial)
28. Packard M, Jokhi V, **Ding B** and Budnik V. (2015) Nucleus to Synapse Nesprin Railroad Tracks Direct Synapse Maturation through RNA localization. **Neuron**. 86(4):1015-28).
29. **Ding B***. (2015) Gene Expression in Maturing Neurons: Regulatory Mechanisms and Related Neurodevelopmental Disorders. **ACTA PHYSIOLOGICA SINICA (Sheng Li Xue Bao)**. 67(2):113-33. (Invited Review)
30. **Ding B**, Wang W, Selvakumar T, Xi HS, Zhu H, Chow CW, Horton JD, Gronostajski RM and Kilpatrick DL. (2013) Temporal Regulation of Nuclear Factor One Occupancy by Calcineurin/NFAT Governs a Voltage-Sensitive Developmental Switch in Late Maturing Neurons. **J Neurosci**. 33(7):2860-2872.
31. **Ding B** and Kilpatrick DL. (2013) Lentiviral Vector Production, Titration, and Transduction of Primary Neurons. **Methods Mol Biol**. 1018:119-31. Chapter 12.
32. **Ding B** and Kilpatrick DL.(2013). Chromatin Immunoprecipitation Assay of Brain Tissue Using Percoll Gradient-Purified Nuclei. **Methods Mol Biol**. 1018:199-209. Chapter 19.
33. **Ding B**, Lejeune D and Li S. (2010) The C-terminal Repeat Domain of Spt5 Plays an Important Role in Suppression of Rad26-independent Transcription Coupled Repair. **J Biol Chem**. 285 (8): 5317-5326.
34. Chen X, **Ding B**, Lejeune D, Ruggiero C and Li S. (2009) Sumoylation of Rpb1 in Response to UV Radiation or Impairment of Transcription Elongation in Yeast. **PLoS One**. 4 (4) e5267.
35. Lejeune D, Chen X, Ruggiero C, Berryhill S, **Ding B** and Li S. (2009) Yeast Elc1 Plays an Important Role in Global Genomic Repair but not in Transcription Coupled Repair. **DNA repair (Amst)**. 8(1): 40-50.
36. **Ding B**, Ruggiero C, Chen X and Li S. (2007) Tfb5 is Partially Dispensable for Rad26 Mediated Transcription Coupled Nucleotide Excision Repair in Yeast. **DNA Repair (Amst)**. 6 (11): 1661- 1669.
37. Li S, **Ding B**, LeJeune D, Ruggiero C, Chen X and Smerdon MJ. (2007) The Roles of Rad16 and Rad26 in Repairing Repressed and Actively Transcribed Genes in Yeast. **DNA Repair (Amst)**. 6 (11): 1596-1606.

38. Li S, **Ding B**, Chen R, Ruggiero C and Chen X. (2006) Evidence that Transcription Elongation Function of Rpb9 is Involved in Transcription Coupled DNA Repair in *Saccharomyces cerevisiae*. **Mol Cell Biol.** 26 (24): 9430-9441.
39. Li S, Chen X, Ruggiero C, **Ding B** and Smerdon M. (2006) Modulation of Rad26 and Rpb9 Mediated DNA Repair by Different Promoter Elements. **J. Biol. Chem.** 281(48): 36643-36651.
40. **Ding B**, Smith ES, and Ding H. (2005) Mobilization of Iron Center in IscA for Iron-sulfur Cluster Assembly in IscU. **Biochem. J.** 389 (Pt 3):797-802.
41. **Ding H**, Clark RJ and **Ding B.** (2004) **IscA Mediates Iron Delivery for Assembly of Iron-Sulfur Clusters in IscU under the Limited Accessible Free Iron Conditions.** **J. Biol. Chem.** 279 (36): 37499 – 37504.

Book Editor

Baojin Ding (Volume Editor): *Human Induced Pluripotent Stem Cells*. Neuromethods series. **Springer Nature**. (In preparation)

Social Media

Baojin Ding. *Patient-specific neurons key in new dystonia discoveries.* **Research Features.** January 31, 2022

[Patient-specific neurons key in new dystonia discoveries \(researchfeatures.com\)](https://researchfeatures.com/patient-specific-neurons-key-in-new-dystonia-discoveries)

TEACHING

• 2022- present

IDSP118, Cell Biology and Signaling (Course directors, Dr. Shile Huang and Dr. Christopher Schmoutz)

Five Lectures each fall semester:

Sept 26 Mon, Second Messengers I	Ding
Sept 27 Tu, Second Messengers II	Ding
Nov 2 Nuclear Pore and Nucleocytoplasmic Transport	Ding
Nov 21 Extracellular Matrix 1	Ding
Nov 22 Extracellular Matrix 2	Ding

Q10. Overall Evaluation (By students)

	Outstanding	Excellent	Good	Fair	Poor	Total
Dr. Baojin Ding	100.00%	3	0.00%	0	0.00%	0
			0.00%	0	0.00%	0
						3
						3
						2

PHARM 204, Brain Research through Advanced and Innovative Neurotechnologies (BRAIN) (Course Director, Dr. Xiao-Hong Lu)

Two Lectures each fall semester:

L25 Human iPS cells to model neurological disorders I	Ding
L26 Human iPS cells to model neurological disorders II	Ding

• Fall Semester (2018-2021)

Note: Dr. Baojin Ding was the sole instructor throughout the whole semester for these courses.

BIOL-457(G), Advanced Cell Biology, CRN 224133, 3 Credits Lecture (40 Hours lectures + 4 exams)

This course aims to examine life at its most fundamental level, including mechanisms and

pathways responsible for membrane transport, metabolism, gene expression, protein synthesis and secretion, membrane trafficking, cytoskeleton dynamics, and cell signaling.

Enrollment: 2018: 35 (31 undergraduate students and 4 graduate students)

2019: 41 (34 undergraduate students and 7 graduate students)

2020: 40 (38 undergraduate students and 2 graduate students)

2021: 40 (35 undergraduate students and 5 graduate students)

Course evaluation by students

Year	Overall Course		Overall Instructor		N (students)	
	Score	Average Score	Score	Average Score	Score	Average Score
2018	3.9	4.0	4.2	4.1	27	8957
2019	4.1	4.0	4.5	4.5	32	7172
2020	4.5	3.9	4.4	4.0	19	7419

Above average

BIOL-458(G), Advanced Cell Biology Lab, 2 Credits, 4 Hours Lab

This class is intended to immerse students in the process of performing scientific experiments, through which students will master some experimental skills, acquire the capabilities for data analysis and result interpretation. The model system will be cultured mammalian cells and/or fixed cells on slides.

Enrollment: 2018: 14 (13 undergraduate students and 1 graduate student)

2019: 21 (19 undergraduate students and 2 graduate students)

2020: 21 (20 undergraduate students and 1 graduate students)

2021: 15 (14 undergraduate students and 1 graduate students)

BIOL-410, 1-6 Credit(s), Individual Project for undergraduate students

Cellular and Molecular Neuroscience - from Basic Knowledge to Advanced Techniques

- Spring Semester (2019-2021)**

Note: Dr. Baojin Ding was the sole instructor throughout the whole semester for these courses.

BIOL-472(G), Neurophysiology, 3 Credits Lecture

(30 Hours lectures + 15 hours discussion + 3 exams).

This course aims to understand neurophysiological activities at cellular and molecular levels and their linkage to human neurological diseases.

Enrollment: 2019: 20 (15 undergraduate students and 5 graduate students)

2020: 14 (8 undergraduate students and 6 graduate students)

2021: 14 (9 undergraduate students and 5 graduate students)

Course evaluation by students

Year	Course Score (n=students)			Instructor score (n=students)		
	Undergraduate	graduate	Average	Undergraduate	graduate	Average
2019	4.3 (12)	4.5 (4)	4.1 (6553)	4.8 (12)	5.0 (4)	4.2 (6517)
2020	4.3 (6)	4.6 (5)	4.0 (5316)	4.3 (6)	4.8 (5)	4.1 (5393)
2021	4.5 (12)	N/A	4.0 (6029)	4.6 (12)	N/A	4.1 (6034)

Above average

BIOL-473(G), Neurophysiology Lab, 1 Credit, 2 Hours Lab

This class aimed to immerse students in the process of performing scientific experiments, through which students will master some experimental skills (such as immunocytochemistry and fluorescent microscopy imaging and analysis), acquire the capabilities for data analysis and result interpretation. The model system will be cultured reprogrammed neurons.

Enrollment: 2021: 9 (5 undergraduate students and 4 graduate students)

BIOL-410, 1-6 Credit(s), Individual Project for undergraduate students
Cellular and Molecular Neuroscience - from Basic Knowledge to Advanced Techniques.

- **Teaching Assistant (2002-2003)**
Wenzhou Medical University, Wenzhou, Zhejiang, China
Course: Molecular Biology (Lecture and Lab)

STUDENTS/POSTDOCS MENTORED (*co-authors of publication)

Postdoc Researcher/Visiting Scholar Mentored

Present:

2020- ***Dr. Masood Sepehrimanesh**, Ph.D., D.V.M., Shiraz University of Medical Sciences, Shiraz, Iran. Postdoctoral Research Associate. LSU Health Shreveport.
Research topic: Determining the pathogenesis of the movement disorder Amyotrophic Lateral Sclerosis using patient-derived motor neurons.

Past:

2018-19 **Dr. Ameneh Zare-Shahabadi**, M.D. Tehran University of Medical Sciences, Iran. Research Assistant. University of Louisiana at Lafayette.
Research topic: Impaired nucleocytoplasmic transport in aging.
Current: Medical Doctor, Cincinnati, OH.

Graduate Students (Serve as Advisory Committee Chair and the Major Advisor)

Present:

01/2021- ***Md Abir Hosain**, PHD graduate student, Biochemistry and Molecular Biology graduate program, LSU Health Shreveport.
Research topic: Contributions of DNA damage and response to neurodegeneration.

01/2020- ***Haochen (Luke) Cui**, PHD graduate student, Biochemistry and Molecular Biology graduate program, LSU Health Shreveport.
Research topic: Roles of Torsin AAA+ ATPase in the regulation of nucleocytoplasmic transport.

08/2019- ***Masuma Akter**, PHD graduate student, Environmental and Evolutionary Biology graduate program, University of Louisiana at Lafayette.
Research topic: Modeling DYT1 dystonia using patient-derived cholinergic neurons.

Past:

2020-22 ***Casey Coutee**, Master graduate student, Environmental and Evolutionary Biology graduate program, University of Louisiana at Lafayette.
Thesis: Abnormal Nuclear Envelope Morphology and Nucleocytoplasmic Transport in Aged Human Cells.
Current: Manufacturing Chemist, Nucleic Acid Solutions Division, Agilent Technologies. Boulder, Colorado.

2019-21 **Jacob Stagra**, PHD graduate student, Environmental and Evolutionary Biology graduate program, University of Louisiana at Lafayette.
Research topic: Effects of dysregulation of nuclear Lamin B1 on nuclear envelope morphology and nucleocytoplasmic transport.
Current: PHD student in Dr. Karen Smith Lab at UL Lafayette.

2019-20 **Makaila Mitchell**, non-thesis Master graduate student, University of Louisiana at Lafayette.
Current: n/a

Graduate Students (Serve as Advisory Committee Member)

Present:

- 07/2022- **Lin Li**, PHD graduate student, Biochemistry and Molecular Biology graduate program, LSU Health Shreveport.
Chair: Dr. Xiuping Yu.
Research topic: The involvement of TET proteins in neuroendocrine differentiation in prostate cancer.
- 08/2019- **Jackie Barua**, PHD graduate student, Environmental and Evolutionary Biology graduate program, University of Louisiana at Lafayette.
Chair: Dr. Karen Smith.
Research topic: Role of amygdala circuitry in self-injurious behavior.

Past:

- 2018-22 **Santosh Paudel**, PHD graduate student, Environmental and Evolutionary Biology graduate program, University of Louisiana at Lafayette.
Chair: Dr. Rirwij Kulkarni.
Dissertation: Defining the roles of bacterial metabolism, virulence, and host NLRP3 inflammasome in Methicillin Resistant *Staphylococcus aureus* urinary tract infection.
- 2018-21 **Brittany M. Pitrucha**, Master graduate student, Environmental and Evolutionary Biology graduate program, University of Louisiana at Lafayette.
Chair: Dr. Karl Hasenstein.
Thesis: The Biological Response of *Raphanus sativus* to Ion Beam Irradiation
- 2018-21 **Alex Esteve**, Master graduate student, Environmental and Evolutionary Biology graduate program, University of Louisiana at Lafayette.
Chair: Dr. Karen Smith.
Thesis: Impacts of FGFR1 and Diet on Hypothalamic Tanycytes and the Hippocampus.
- 2019-21 **Bria Johnson**, Master graduate student, Environmental and Evolutionary Biology graduate program, University of Louisiana at Lafayette. (Non-thesis)
Chair: Dr. Rirwij Kulkarni.
- 2018-20 **Chukwunonso Chukwudozie**, Master graduate student, Environmental and Evolutionary Biology graduate program, University of Louisiana at Lafayette.
Chair: Dr. Francois Villinger.
Topic: Developing and Optimisation of SIV PET probes able to image SIV in the Central Nervous System.

Undergraduate and High School Students

- Summer 2023 **Amarige Yusufji**, Texas Christian University in Fort Worth, Undergraduate Research Apprenticeship Program (UGRAP), LSU Health Shreveport.
Research topic: Impact of Nuclear Lamina Protein Levels on Nuclear Morphology.
- Summer 2023 **Cameron Wilson**, Captain High School Shreveport, Jumpstart Summer Enrichment Program (JSEP), LSU Health Shreveport.
Research topic: Evaluation of neurodevelopment via measuring neurite outgrowth of reprogrammed human neurons.
- Summer 2022 **Frankie Parratt**, Caddo Magnet High, Jumpstart Summer Enrichment Program (JSEP), LSU Health Shreveport.
Research topic: Direct Conversion of Adult Fibroblasts into Motor Neurons.
- 01/2020-12/2021 **Catherine Wertz**, Scholarship undergraduate Researcher, Biology major, UL Lafayette. She completed her undergraduate student thesis.
Research topic: Confocal image analysis to measure neurodevelopment.
- 08/2018-12/2020 **Bennett Garbarino**, Scholarship undergraduate Researcher, Biology major, UL Lafayette.

- 08/2018-05/2020 **Matthew Authement**, Scholarship undergraduate Researcher, Biology major, UL Lafayette.
- 01/2019-05/2020 ***Casey A. Coutee**, Undergraduate Researcher, Biology major, UL Lafayette. BIOL-410 Individual Project. He joined lab as a master graduate student in Fall 2020.
Research topic: Nuclear envelope morphology analysis of human fibroblast cells using immunocytochemistry of nuclear Lamins.
- 08/2019-05/2020 **Bao Doan**, Undergraduate Researcher, Biology major, UL Lafayette. BIOL-410 Individual Project. Cellular and Molecular Neuroscience - from Basic Knowledge to Advanced Techniques.
- 08/2019-12/2019 **Audrey Lumpki**, Undergraduate Researcher, Biology major, UL Lafayette. BIOL-410 Individual Project. Cellular and Molecular Neuroscience - from Basic Knowledge to Advanced Techniques.
- 08/2019-05/2020 **Erin Miller**, Undergraduate Researcher, Biology major, UL Lafayette. BIOL-410 Individual Project. Cellular and Molecular Neuroscience - from Basic Knowledge to Advanced Techniques.

PROFESSIONAL ACTIVITIES

Professional Services

Associate Editor

Frontiers in Molecular Neuroscience (2018-) (IF=6.261)

As of June 2023, edited 12 manuscripts as a Handling Editor (6 published and 6 rejected). Reviewed 32 manuscripts as a reviewer editor (29 published and 3 rejected).

Editorial Board

American Journal of Psychiatry and Neuroscience (AJPN) (2020-); (IF=5.699)

Austin Neurology (2016-2018);

Journal of Autism & Related Disabilities (2016-2018);

Journal of Down Syndrome & Chromosome Abnormalities (2015-2017);

Frontiers in Molecular Neuroscience (2013-2018)

Grant Reviewer

DoD Scientific Reviewer Neurological Disorders (ND) Panel (2021)

European Research Council (ERC) grant review (2016)

Ad Hoc Reviewer:

2023:

FEBS Letters

Cells (4)

Frontiers in Molecular Neuroscience

Current Molecular Medicine (CMM)

2022:

Antioxidants

Cells (2)

Cellular and Molecular Life Sciences (CMLS)

Life Sciences in Space Research (LSSR)

Dystonia

Redox Biology

Neural Regeneration Research (NRR)

2021:

Cells

JoVE

Neural Regeneration Research (NRR) (3)

2020:

International Journal of Molecular Sciences (IJMS)

Neural Regeneration Research (NRR)

Prior to 2020:

Neurobiology of Disease

Scientific Reports

Journal of Cellular Biochemistry

Journal of Cellular and Molecular Medicine

Frontiers in Molecular Neuroscience

Analytica Chimica Acta (ACA)

Frontiers in Computational Neuroscience

Journal of Neurosciences in Rural Practice

etc.

Scientific Member

Member of International Society for Stem Cell Research (ISSCR) (2022-)
Member of American Society for Cell Biology (ASCB) (2017-)
Member of Society for Neuroscience (2014-)
Member of American Association of Clinical Anatomists (AACA) (2012-2016)
Member of American Association for the Advancement of Science (AAAS) (2009-2013)
Member of American Society for Microbiology (ASM) (2009-2012)
Member of Environmental Mutagenesis Society (EMS) (2009-2010)

University/Department Services

Graduate Admission Committee, BMB department LSUHS 2023-
Summer Pipeline Mentors, LSUHS 2023
Medical Student Research Poster Presentation- Judging, LSUHS 2022
Summer Pipeline Mentors, LSUHS 2022
Grant Review: Predoctoral Review Committee, LSUHS, Spring 2022
Judge Postdoctoral Fellow Poster on Graduate Research Day, LSUHS, Spring 2022
Faculty Search Committee for Physiologist, UL Lafayette (2019-2020)
Pre professional Committee, UL Lafayette (2020- 2022)

Public Services

Louisiana Region VI Science and Engineering Fair Judge 2019
Massachusetts State Middle School Science and Engineering Fair Judge (2014-2016)

Reuters Health

<http://www.psychcongress.com/article/tiny-brain-organoids-show-neuron-differentiation-autism-23405>

ORAL PRESENTATIONS AND INVITED TALKS (in recent five years)

1. Scheduled: November 11, 2023. *Modeling movement disorders using patient-specific motor neurons*. The 2nd Annual NIH Investigator Meeting for Interoception Research. November 11, 2023. Bethesda, Maryland. (In Person)
2. April 27, 2023. *Patient-specific neurons are key to new discoveries in neurological diseases*. The Global Experts meet on Neurology, Neuroscience and Brain Disorders. April 27-29, 2023. Paris, France. (Invited Speaker, In Person)
3. October 28, 2022. *Modeling Human Neurological Diseases Using iPSC-derived Neurons*. Department of Biological Sciences, Louisiana State University-Shreveport. (Invited talk. In Person. Host: Dr. Stephen W. Banks)
4. October 21, 2022. *Nucleocytoplasmic Transport and Its Linkage to Neurological Diseases*. Department of Environmental Health Science, University of Georgia College of Public Health. Athens, GA 30602. (Invited talk. Virtual. Host: Dr. Wentao Li)
5. June 15, 2022. *Modeling Neurological Diseases Using Patient-derived Neurons*. The 5th Edition of International Conference on Neurology and Neurological Diseases. June 15-16, 2022. (Invited Speaker. Virtual)
6. July 9, 2021. *Nucleocytoplasmic Transport and Its Linkage to Neurological Diseases*. Department of Anatomy, Howard University College of Medicine, Washing, D.C. (Invited talk. Virtual. Host: Dr. Antonei Csoka)
7. May 3, 2021. *Nucleocytoplasmic Transport and Its Linkage to Aging and Neurological Diseases*. Department of Neuroscience & Regenerative Medicine, Medical College of Georgia at Augusta University. Augusta, GA (Invited Talk. Virtual. Host: Dr. Dave Blake)
8. April 28, 2021. *Nucleocytoplasmic Transport and Its Linkage to Aging and Neurological Diseases*. Sponsored by the Dept. of Biochemistry & Molecular Biology and the Oklahoma Center for

GeroScience and Healthy Brain Aging. The University of Oklahoma Health Sciences Center (OUHSC), Oklahoma City, OK. (Invited Talk. Virtual. Host: Drs. Ann Olson and William Sonntag)

9. February 23, 2021. *Nucleocytoplasmic Transport and Its Linkage to Neurological Diseases*. Department of Cell Biology and Anatomy, LSU Health Shreveport, Shreveport, LA (Virtual. Host: Dr. Kevin McCarthy)
10. January 5, 2021. *Impaired Nucleocytoplasmic Transport in Neurological Diseases*. Department of Biochemistry and Molecular Biology, LSU Health Shreveport, Shreveport, LA (Virtual. Host: Dr. Stephan N. Witt)
11. January 23, 2020. *Impaired Nucleocytoplasmic Transport in Neurological Diseases*. Department of Biological Sciences, University of Texas at Dallas, Dallas, TX (Invited Talk. In Person. Host: Dr. Heng Du)
12. July 19, 2019. *Nucleocytoplasmic Transport in Neurodegenerative Diseases*. National Clinical Research Center for Geriatric Disorders, Xiangya Hospital, Central South University, Changsha, Hunan Province, China. (Invited Talk. In Person. Host: Dr. Yu Tang)
13. May 7, 2018. *Spatiotemporal Regulation of Gene Expression*. Department of Biology, University of Louisiana at Lafayette, Lafayette, LA. (In Person. Host: Dr. Karl H. Hasenstein)

POSTER PRESENTATIONS (in recent five years)

1. Scheduled: November 10-15, 2023. **Ding B.** *Modeling movement disorders using patient-specific motor neurons*. Neuroscience Annual meeting. Washington DC. November 10-15, 2023. (In person)
2. December 4, 2022. **Ding B.** *Impaired Nucleocytoplasmic Transport in Neurological Diseases*. American Society for Cell Biology (ASCB) Annual Meeting. Washington DC. December 3-7, 2022. (In person)
3. November 9, 2022. **Ding B.** *Similarities and differences of reprogrammed neurons via direct conversion and human induced pluripotent stem cells in modeling DYT1 dystonia*. Neuroscience 50th Annual Meeting (Virtual: November 8-11, 2022).
4. October 20, 2022. Luke Cui and **Baojin Ding**. *The Pathogenesis of Torsin ATPase mutation (ΔE) in the movement disorder Dystonia*. Biomedical Research And Industry Day (BRAID). LSU-Shreveport. One University Place, Louisiana. (In person)
5. June 8, 2021. **Ding B.** *Modeling Neurodegenerative Diseases via Generation of Patient-specific Neurons*. Virtual Keystone Symposia. Neurodegenerative Diseases: Genes, Mechanisms and Therapeutics. June 7-9, 2021.
6. December 3, 2020. **Ding B.**, Yu T. and Zhang C-L. *Dysregulated nuclear LMNB1 and impaired nucleocytoplasmic transport in Dystonia patient-derived neurons*. The American Society for Cell Biology (ASCB) and Annual Meeting. December 2-16, 2020. ASCB/EMBO Virtual Meeting.
7. K Selvam, **B Ding**, R Sharma and S Li. *Promotion of error-free transcriptional bypass of DNA lesions is essential for Rad26 to facilitate transcription coupled DNA repair*. ENVIRONMENTAL AND MOLECULAR MUTAGENESIS 59, 71-71, 2018.

OTHER PRESENTATIONS

8. Journal Club. March 8, 2023
Paper presented: Otsuka S. ... and Ellenberg J. A quantitative map of nuclear pore assembly reveals two distinct mechanisms. Nature. 2023 Jan; 613(7944):575-581.
9. Journal Club. May 25, 2022.
Paper presented: Guan J. ...and Deng H. Chemical reprogramming of human somatic cells to pluripotent stem cells. Nature. 2022 May; 605(7909):325-331.

ABSTRACTS AND PRESENTATIONS GIVEN BY TRAINEES

1. July 28, 2023. **Cameron Wilson**, Masood Sepehrimanesh and Baojin Ding. Evaluation of neurodevelopment via measuring neurite outgrowth of reprogrammed human neurons. (Poster)
2. July 27, 2023. **Sunny Yusufji**, Md Abir Hosain, Masood Sepehrimanesh and Baojin Ding. Impact of Nuclear Lamina Protein Levels on Nuclear Morphology. (Poster)
3. July 21, 2023. **Masood Sepehrimanesh**. Generation of patient-specific motor neurons in modeling movement disorder amyotrophic lateral sclerosis. Graduate Research Day, LSU Health Shreveport. **(The only representative was selected for the oral presentation from the Postdoctoral category)**
4. July 21, 2023. **Luke Cui**, Md Abir Hosain and Baojin Ding. Proteomic study and AlphaFold2 prediction reveal DYT1 dystonia-causing mutation Tor1A- ΔE disrupts nucleocytoplasmic transport through trapping the major transport receptor exportin 1 (Xpo1) in patient-derived neurons. Graduate Research Day, LSU Health Shreveport. **(Won The First Place of Poster Presentation in Junior category)**.
5. July 21, 2023. **Md Abir Hosain** and Baojin Ding. Determining the pathogenesis of the movement disorder ALS4 using reprogrammed human motor neurons. Graduate Research Day, LSU Health Shreveport. (Poster)
6. May 04, 2023. **Md Abir Hosain**. *Direct conversion of human motor neurons in modeling movement disorders*. Department of Biochemistry and Molecular Biology Seminar Series, LSU Health Shreveport. (Oral)
7. January 19, 2023. **Masuma Akter**. *Patient-derived neurons reveal dysregulation of cholinergic system in dystonia*. Department of Biochemistry and Molecular Biology Seminar Series, LSU Health Shreveport.
8. September 22, 2022. **Masood Sepehrimanesh**. *Identification of dysregulated factors in patient iPSC-derived neurons*. Department of Biochemistry and Molecular Biology Seminar Series, LSU Health Shreveport. (Oral)
9. July 29, 2022. **Frankie Parratt**, Masuma Akter, Masood Sepehrimanesh, and Baojin Ding. *Direct Conversion of Adult Fibroblasts into Motor Neurons*. Jumpstart Summer Enrichment (JSEP), 2022 Summer Pipeline, LSU Health Shreveport. (Poster)
10. July 02, 2021, **Masuma Akter**. *Modeling movement disorder DYT1 dystonia by using human-specific neurons*. Proposal defense. Department of Biology, University of Louisiana at Lafayette. (Oral)
11. October 20, 2022. **Luke Cui** and Baojin Ding. *The Pathogenesis of Torsin ATPase mutation (ΔE) in the movement disorder Dystonia*. Biomedical Research And Industry Day (BRAID). LSU-Shreveport. One University Place, Louisiana. (Poster)
12. May 6, 2022. **Luke Cui** and Baojin Ding. *A new TorsinA role in Nucleocytoplasmic transportation of DYT1 Dystonia*. Graduate Research Day. LSU Health Shreveport. (Poster)
13. February 18, 2021. **Luke Cui**. *Nucleo-Cytoplasmic transport defects in DYT1 dystonia*. Department of Biology colloquium series, University of Louisiana at Lafayette. (Oral)
14. February 11, 2021, **Masuma Akter**. *Modeling of neurological movement disorder dystonia by using human specific neurons*. Department of Biology colloquium series, University of Louisiana at Lafayette. (Oral)
15. November 11, 2020, **Masuma Akter**. *Generation of human neurons from fibroblast for disease modeling*. 3 Minute Thesis (3MT), University of Louisiana at Lafayette. (Oral)
16. October 15, 2020, **Masuma Akter**. *Generation of human neurons from fibroblast for disease modeling*. Department of Biology colloquium series, University of Louisiana at Lafayette. (Oral)

17. April 26, 2019. **Casey Coutee**, Bennett Garbarino, and Baojin Ding. *Abnormal Nuclear Morphology and Nucleocytoplasmic Transport during Aging*. Biology Undergraduate Research Symposium (BURS), Department of Biology, UL Lafayette. (Poster)
18. July 26, 2017. **Joy Aniede**, Baojin Ding, and Chun-Li Zhang. *Nucleocytoplasmic transport in aging and neurodegenerative disorders*. Science Teacher Access to Resources at Southwestern (STARS), Summer Research Program, UT Southwestern Medical Center. (Poster)

LINKS

[Visit the Ding Lab](#) at LSU Health Shreveport

Google Scholar Profile

<https://scholar.google.com/citations?user=gGuQKNIAAAAJ&hl=en>

Research Features: Baojin Ding. *Patient-specific neurons key in new dystonia discoveries*. [PDF](#)

Reuters Health <http://www.psychcongress.com/article/tiny-brain-organoids-show-neuron-differentiation-autism-23405>