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hiPSC-NPCs: Differentiation of NPCs from hiPSCs

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We use this protocol and it's working

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Abstract

We present a method by which to efficiently differentiate hiPSCs into NPCs, which in addition to being capable of further differentiation into functional neurons, can also be robustly passaged, freeze-thawed or transitioned to grow as neurospheres, enabling rapid genetic screening to identify the molecular factors that impact cellular phenotypes including replication, migration, oxidative stress and/or apoptosis. Patient derived hiPSC NPCs are a unique platform, ideally suited for the empirical testing of the cellular or molecular consequences of manipulating gene expression.
An update of the protocol published here: **https://pubmed.ncbi.nlm.nih.gov/25742222/**

Materials

- Neural Crest Stem Cell MicroBeads human (Miltenyi Biotech, 130-097-127)
- Indirect CD133 MicroBeads human (Miltenyi Biotech, 130-091-895)
- autoMACS Running Buffer - MACS Separation Buffer (Miltenyi Biotech, 130-091-221)
- LS Columns (order #130-042-401)
- LD Columns (order #130-042-901)
- MidiMACS separator Starting Kit w/LS columns (order #130-042-301)

Media Components

N2B27 Media
- DMEM F12 GlutaMAX (Invitrogen #10565)
- N2 (Invitrogen #17502-048) 1X
- B27 w/o vit A (Invitrogen #12587-010) 1X
- LDN193189 (Stemgent 193189) 0.1 uM
- SB (Tocris 431542) 10uM

NPC media
- DMEM F12 GlutaMAX (Invitrogen #10565)
- N2 (Invitrogen #17502-048) 1X
- B27 w/o vit A (Invitrogen #12587-010) 1X
- FGF2 (Invitrogen) 20ng/mL
- Laminin (Invitrogen 23017-015) 1mg/mL

NPC specific Markers
- SOX1
- SOX2 : a *transcription factor* that is essential for maintaining self-renewal, or *pluripotency_, of undifferentiated embryonic stem cells_. Sox2 has a critical role in maintenance of embryonic and neural stem cells*
- Nestin
- Vimentin

Antibodies
- Goat anti-Sox2, 1:200
- Mouse anti-human Nestin (Chemicon), 1:200
- Rabbit anti-βIII-tubulin (Covance), 1:200
- Mouse anti-βIII-tubulin (Covance), 1:500
- Rabbit anti-MAP2AB, 1:200
- Rabbit anti-Sox2 (Santa Cruz), 1:200
- Rabbit anti-Ki67 (Abcam), 1:500
- Rabbit anti-TBR2 (Abcam), 1:100
- Mouse anti-Vimentin (MBL International), 1:200
- Rabbit anti-Pax6 (Covance), 1:200

- Anti-mouse MTC02 (Abcam ab3298), 1:500

Safety warnings

Be careful not to break up cell clumps with excessive pipetting
Be careful to not break up any rosette aggregate

Before start

- Prepare and warm N2/B27 media
- Prepare and warm NPC media
- Prepare Matrigel coated plates before the beginning of every split required by at least 1 hour.

Procedure

1 Grow hiPSCs on Matrigel and before splitting by 1 day dissociate colonies using EDTA.

2 Aspirate hES media from 1 well (from 6 well plate) and wash with 1mL PBS.

3 To each well add 1mL 0.5mM EDTA and incubate for 3-5 minutes at room temperature (Small holes should start appearing at the edges of colonies which is an indication of dissociation).

4 Following incubation, most cells are still attached to the plate surface. Carefully aspirate EDTA and wash cell clumps off the plate using 3ml in N2/B27 media.

5 After suspending the cell clumps in 3mL of media, carefully collect cell clumps using a 5mL strip pipette and transfer to 1 well of 6 well Low attachment plates.

6 Gently shake the plate from side to side to break up any fragments sticking to each other and incubate the plate overnight at 37°C to allow for EBs formation.

7 Prepare a 15mL Falcon tube with 5mL DMEM.

8 Gently collect EBs with a 10mL strip pipette and add it to the 5mL DMEM wash.

9 Let EBs sink to the bottom and then aspirate DMEM.

10 Resuspend EBs in 3mL N2/B27 Media.

11 Using a 5mL strip pipette, collect the EBs suspension and add it back to the plate.

12 Into Porn-laminin coated plates, add 2mL of N2/B27 media.

13 Transfer floating EBs to the plates and allow them to settle and adhere.

14 Aspirate media from plates to collect any EBs that haven't settled.

15 Add 2mL of N2/B27 Media.

16 Full media change using 2mL of N2/B27 media.

17 Full media change using 2mL of N2/B27 media.

17.1 Aspirate media from wells and add 1mL of NRSR per well.

17.2 Incubate at 37°C for 1 hour.

17.3 Using a p1000 pipet, gently remove the enzyme from each well and add 1mL of DMEM.

17.4 Using a p1000 pipet, collect 1mL of DMEM and expel the DMEM back into the well to detach the rosettes from the plate.

17.5 Collect the rosettes' suspension and add to a 15mL falcon tube.

17.6 Repeat Step 4 26 5 to detach any remaining rosettes*. Add the suspension to the same falcon tube. If the rosettes don't detach readily, you may add more enzyme and repeat the process.

18 Spin cells at 300g for 3 minutes

19 Aspirate supernatant and resuspend rosettes in 2mL of NPC media.

20 Transfer cells (1:1) into Matrigel plates.

20.1 Manually pick neural rosettes to a 6 well matrigel coated plate.

20.2 At Day 20 as well, do a second round of picking.

20.3 Manually select the best of the picked rosettes and add it to a new matrigel coated well with 1mL of Accutase.

20.4 Incubate for 15 minutes at 37°C.

20.5 Spin cells at 300g for 3 minutes.

20.6 Aspirate supernatant and re-suspend rosettes in 2mL of NPC media.

20.7 Transfer cells into a 24 well matrigel coated plate.

NPC Sorting

21 Determine cell number.

22 Centrifuge cell suspension at 400×g for 10 minutes. Aspirate supernatant completely.

23 Resuspend cell pellet in 80 µL of buffer per 10^7 total cells.

24 Add 20 µL of Neural Crest Stem Cell MicroBeads per 10^7 total cells.

25 Mix well and incubate for 15 minutes in the refrigerator (2–8 °C).

26 Resuspend up to 10^8 cells in 500 µL of buffer.

27 Proceed to magnetic separation.

Depletion with LD Columns

28 Place LD Column in the magnetic field of a suitable MACS Separator. For details refer to the LD Column data sheet.

29 Prepare column by rinsing with 2 mL of buffer.

30 Apply cell suspension onto the column.

31 Collect unlabeled cells that pass through and wash column with 2×1 mL of buffer. Collect total flow-through; this is the unlabeled cell fraction. Perform washing steps by adding buffer two times. Only add new buffer when the column reservoir is empty. Do not use the plunger.

Magnetic labeling with Indirect CD133 MicroBeads

32 Determine cell number.

33 Centrifuge cell suspension at 400×g for 4 minutes. Aspirate supernatant completely.

34 Resuspend cell pellet in 350 µL of buffer per 10^8 total cells.

35 Add 100 µL of FcR Blocking Reagent per 10^8 total cells.

36 Add 50 µL of CD133/1 (AC133)-Biotin per 10^8 total cells.

37 Mix well and refrigerate for 10 minutes (4–8 °C).

38 Wash cells by adding 10–20× the labeling volume of buffer and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.

39 Repeat washing step.

40 Resuspend up to 10^8 total cells in 400 µL of buffer.

41 Add 100 µL of Anti-Biotin MicroBeads per 10^8 total cells.

42 Mix well and refrigerate for 15 minutes (4–8 °C).

43 Wash cells by adding 10–20× the labeling volume of buffer and centrifuge at 400×g for 4 minutes. Aspirate supernatant completely.

44 Resuspend up to 10^8 cells in 500 µL of buffer.

45 Proceed to magnetic separation.

Magnetic separation with LS Columns

46 Place column in the magnetic field of a suitable MACS Separator.

47 Prepare column by rinsing with 3mL of buffer in LS column.

48 Apply cell suspension onto the column.

49 Collect unlabeled cells that pass through and wash column with 3mL of buffer. Perform washing steps by adding buffer three times with 3mL. Only add new buffer when the column reservoir is empty.

50 Collect total effluent. This is the unlabeled cell fraction.

51 Remove column from the separator and place it on a suitable collection tube.

52 Pipette an appropriate amount of buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column. LS: 5 mL

53 To increase the purity of CD133+ cells, the eluted fraction can be enriched over a second column. Repeat the magnetic separation procedure as described in steps 1 to 6 by using a new column.

Media Components

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Supplements	Catalogue #	Final Volume	Volume to add
DMEM F12 GlutaMAX	Invitrogen #10565		
N2	Invitrogen #17502-048	1X	
B27 w/o vit A	Invitrogen #12587-010	1X	
LDN193189	Stemgent 193189	0.1 uM	
SB	Tocris 431542	10uM	

NPC media

Supplements	Catalogue #	Final Volume	
DMEM F12 GlutaMAX	Invitrogen #10565		
N2	Invitrogen #17502-048	1X	
B27 w/o vit A	Invitrogen #12587-010	1X	
FGF2	Invitrogen	20ng/mL	
Laminin	Invitrogen 23017-015	1mg/mL	

Protocol references

https://pubmed.ncbi.nlm.nih.gov/25742222/
https://www.nature.com/articles/mp201422