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Differentiation of PSCs to Neural Progenitors

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Abstract

This protocol describes the differentiation of pluripotent stem cells (PSCs) to neural progenitors.

Guidelines

Definitions

- SMADi- SMAD Inhibitor
- Rocki- Rock Inhibitor
- DMEM- Dubecco’s Modified Eagle Medium
- HBSS- Hank’s Balanced Salt Solution
- NP- neural passage
- SF- StemFlex
- PDL- Poly-D-Lysine

Materials

Reagents:

1. Geltrex (Gibco; A14133)
2. HBSS, no calcium, no magnesium **Thermo Fisher Catalog #**14170070
3. Versene Solution **Thermo Fisher Catalog #**15040066
4. StemFlex Medium **Thermo Fisher Scientific Catalog #**A3349401
5. Rocki (LTK; Y1000)
6. Accutase **Gibco - Thermo Fisher Scientific Catalog #** A1110501
7. N2 supplement **Gibco - Thermo Fisher Scientific Catalog #**17502048
8. DMEM/F-12 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**D6421
9. B-27 Supplement **Gibco - Thermo Fisher Scientific Catalog #**17504044
10. Neurobasal medium **Gibco - Thermo Fisher Scientific Catalog #**21103049
11. GlutaMAX **Gibco - Thermo Fisher Scientific Catalog #**35050038
12. SB431542 (Cell guidance systems; SM33)
13. LDN193189 hydrochloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**SML0559
14. XAV939 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**X3004
15. RevitaCell Suppplement 100X **Thermo Fisher Scientific Catalog #**A2644501
16. Poly-D-Lysine **Thermo Fisher Scientific Catalog #**A3890401
17. L-Ascorbic acid **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**A4403
18. Laminin **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**L2020
19. Trypan Blue **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T8154
20. DAPT **Santa Cruz Biotechnology Catalog #**sc-201315A

Media:

- **N2 media**

	A	B
	DMEM	49ml
	N2 supplement	0.5ml
	Glutamax	0.5ml

- **B27**

	A	B
	Neurobasal medium	48.5ml
	B27	1ml
	Glutamax	0.5ml

Geltrex Coating

2h

1 Slowly thaw a 200 µL aliquot of Geltrex On ice or in the refrigerator until fully thawed.

2 Dilute the thawed Geltrex by adding the 200 µL aliquot to 20 mL of cold DMEM/F12. Keep the mixture On ice during preparation.

3 Immediately add 1 mL /well of a 6-well plate or 100 µL /well of a 96-well plate. Gently swirl the plate(s) to ensure even coating.

4 Incubate the plate(s) at 37 °C for at least 02:00:00 . Prevent the plates from drying during incubation.

2h

5 After incubation, aspirate the unbound Geltrex solution by tilting the plate slightly to avoid disturbing the coated surface. Add the appropriate volume of cell culture media to the coated wells and warm the plate(s) in the incubator prior to adding cells.

Note

Storage of Coated Plates:

If not used immediately, seal the plates with parafilm to prevent dehydration. Store them at 2–8°C for up to 7 days. Before use, let the plate(s) equilibrate to room temperature (15–25°C) for 30 minutes, and then aspirate the Geltrex solution.

Thawing PSCs

1d 0h 2m

6 Prewarm the PSC defrosting medium by adding SF medium + RevitaCell, 1:100 dilution. Use 2 mL per well for a 6-well plate.

7 Ensure a Geltrex-coated well is prepared for each vial of PSCs to be thawed.

8 Place cryovials on dry ice.

9 Quickly thaw a vial in a 37°C water bath until only a small ice pellet remains.

10 Gently add 1 mL of Room temperature defrosting medium to the cryovial. Transfer the diluted cells to a 15 mL tube.

11 Centrifuge the cell suspension at 1250 rpm, 00:02:00 . 2m

12 Carefully aspirate the supernatant and gently resuspend the cell pellet in the prewarmed defrosting medium.

13 Add 2 mL per well of the cell suspension for a 6-well plate using a P1000 micropipette.

14 Ensure the cells are evenly distributed across the well by gently swirling the plate. Place the plate in an incubator (37°C; 5% CO_2; 5% O_2) and allow the cells to recover for 24:00:00 . 1d

15 After 24 hours, replace the defrosting medium with prewarmed StemCell medium. Continue changing the medium daily until cells reach confluence.

Freezing PSCs 1d 0h 5m

16 Cool a Mr. Frosty to 4 °C .

17 Prepare PSC freezing medium by adding 10% DMSO to StemFlex medium. Use 1 mL per well from a 6-well plate.

18 Aspirate the medium from one confluent well of the PSC culture. Rinse the well with 1 mL of HBSS (without calcium and magnesium).

19 Add 1 mL of Versene to the well and swirl the dish to coat the entire surface. Periodically check plates for signs of colonies detaching.

20 Incubate the dish at 37 °C for 00:02:00 – 00:03:00 . 3m

21 Aspirate the Versene and replace it with 1 mL of StemFlex medium per well.

22 Gently dislodge the cells using a cell lifter.

23 Using a P1000 micropipette, transfer the cell suspension to a 15 mL tube containing 1 mL of Room temperature StemFlex medium.

24 Centrifuge the cell suspension at 1250 rpm, 00:02:00 . 2m

25 Label cryovials.

26 Carefully remove the supernatant and resuspend the cells in PSC freezing medium, using 1 mL per well of a 6-well plate.

27 Transfer 1 mL of the suspension into each cryovial and place in a 4 °C Mr. Frosty. Transfer to a -80°C freezer and leave for 24:00:00 . 1d

28 After 24 hours, transfer the cryovials to liquid nitrogen for long-term storage.

Expansion of PSCs 1d 0h 3m

29 Check that the cells are approximately 90% confluent before proceeding.

30 Aspirate the medium from one confluent well of the PSC culture. Rinse the well with 1 mL of HBSS (without calcium and magnesium).

31 Add 1 mL of Versene to the well and swirl the dish gently to coat the entire surface. Periodically check plates for signs of colonies detaching.

32 Incubate the dish at 37 °C for 00:02:00 – 00:03:00 . 3m

33 Aspirate the Versene and replace it with 1 mL of StemFlex medium per well.

34 Gently dislodge the cells using a cell lifter.

35 Using a P1000 micropipette, transfer the cell suspension to a 15 mL tube containing 5 mL of Room temperature StemFlex medium (for 2 wells).

36 Mix the cell suspension by pipetting up and down 3 times with a 10 mL serological pipette.

37 Add 3 mL of the cell suspension to each of 2 wells in a 6-well plate.

38 Gently swirl the plate to ensure the cells are evenly distributed across the wells.

39 Place the plate in an incubator (37 °C ; 5% CO_2; 5% O_2) and allow the cells to recover for 24:00:00 . 1d

Differentiating PSCs to Neural Progenitors: Day -1 1d 0h 5m

40

Note

Plate PSCs in StemFlex medium onto 6-well Geltrex-coated plates, at a density that will ensure cells reach approaching 100% confluence within 24 hours of plating **24 hours prior to desired neuralisation start time**. Cells should be ~60% confluent prior to replating.

See Appendix for details regarding timing - If cells do not reach total confluence by D-1 +3d, discard them. Allowing cells to remain at high confluence for longer periods of time diminishes PSC quality and results in variable growth across the well, as well interfering with the timing of subsequent passages.

PSC passage number must fall between 20 – 30 at the start of neuralisation.

Passaging:

Aspirate total volume of media from each well, and rinse with HBSS at Room temperature (1 mL /well) to remove calcium.

41 Add 1 mL /well Room temperature Versene (EDTA) and incubate (37 °C ; 5% CO_2; 5% O_2) for 00:03:00 - 00:05:00 . Periodically check plates for signs of colonies detaching. 5m

42 Aspirate total volume of Versene and replace with 1 mL /well Room temperature StemFlex media. Working rapidly, use a cell lifter to gently detach colonies from plate.

43 Using a 1000 µL pipette, carefully pipette up and down once (collect cells, pipette suspension across well, collect) to achieve a homogenous suspension of large PSC clusters. Or use a cell lifter to gently detach the cells from the well and then collect the cells with a P1000 pipette.

Note

Breaking the colonies up too much will delay PSCs in reaching 100% confluence.

44 Using the same procedure, collect suspension from all wells into a 50 mL tube. With a 10 mL serological pipette add to existing cell suspension the correct volume of Room temperature StemFlex, such that you have twice the volume of suspension as you do Geltrex coated wells.

45 Aspirate Geltrex and aliquot StemFlex across plates at 1 mL /well.

46 Using a 10 mL serological pipette transfer (a well at a time) 2 mL of the cell suspension to each well.

47 Gently swirl plates to distribute cells evenly across wells and incubate (37 °C ; 5% CO_2; 5% O_2) for 24:00:00 . 1d

48 Following 24-hour incubation, check plates. If cells are not approaching 100% confluent/are loosely packed or appear to have suffered during passage, change media (3 mL /well Room temperature StemFlex) and wait an additional day before neuralisation.

Differentiating PSCs to Neural Progenitors: Day 0 2d

49

Note

Cells must be 100% confluent, or approaching 100% confluence prior to start of neuralisation. Inducing neuralisation while cells are <100% confluent will result in differentiation towards neural crest or nonspecific cells.

Aspirate total volume of StemFlex medium from each well, and replace with 2 mL /well of warm neuralisation medium containing 50% N2/50% B27 media + [M] 0.1 micromolar (µM) LDN193189 + [M] 10 micromolar (µM) SB43 (SMAD inhibitors 2i).

50 Incubate plate for 24:00:00 (37°C; 5% CO_2; 20% O_2). 1d

51 Following 24-hour incubation, aspirate neuralisation medium and replace with 2 mL /well warm neuralisation medium. Incubate plate for 24:00:00 (37°C; 5% CO_2; 20% O_2). Continue replacing medium every 24 hours until **Day 7**, or until formation of a uniform neuroepithelial sheet occurs. 1d

Note

A substantial amount of cell death occurs within the first 24 hours of neuralisation; shake plates to resuspend dead cells prior to aspirating medium on Day 1. Cell layer thickens during initial stage of neuralisation – take care when changing media (especially from ~ Day 5 onwards), as forceful pipetting can cause cell layer to detach from plate.

Differentiating PSCs to Neural Progenitors: Day 7 – np1

2d 0h 7m 30s

52

Note

At this stage cells must be passaged 1:1.

Remove plate(s) from incubator.

53 Aspirate total volume of medium from each well, and rinse with 1 mL /well Room temperature HBSS. Let sit for 00:00:30 .

30s

54 Remove total volume of HBSS and add 1 mL /well Room temperature (4 °C) Accutase (keep protected from light). Immediately transfer plate to incubator and incubate for 00:02:00 – 00:05:00 (37 °C ; 5% CO_2; 20% O_2), until material can be lifted with gentle pipetting.

5m

55 Prepare 15 mL tubes containing Room temperature DMEM at twice the volume of Accutase to be added to the tubes.

56 Using a P1000 micropipette, attempt to lift all cells by pipetting up and down as gently and as little as possible no more than 5 times.

Note

Any remaining attached cells can be collected by rinsing the plate with 1mL/well RT DMEM.

57 Collect cells in Accutase, and transfer directly into DMEM.

Note

Do this one well at a time (i.e. collect cells and transfer to DMEM before detaching cells from adjacent well) to minimize exposure of cells to accutase. Avoid exposing cells to air – do not introduce air bubbles; transfer cells in accutase directly into DMEM. Attempt to produce a homogenous suspension of large cell clusters.

58 Rinse plates with DMEM (1 mL /well). Add to tube(s) containing Accutase/DMEM/cells.

59 Centrifuge cell suspension (1250 rpm, 00:02:00) and carefully remove supernatant, leaving ~ 50 µL atop pelleted cells. 2m

60 Prepare plates. Aspirate Geltrex and aliquot fresh neuralisation medium + [M] 10 micromolar (µM) ROCKi across plates at 1 mL /well.

61 Add Room temperature neuralisation medium + [M] 10 micromolar (µM) ROCKi to tube with cell pellet, at a volume equal to the number of wells passaged. Resuspend by gently pipetting up and down using a 10 mL serological pipette.

Note

If pellet failed to resuspend during step 61, pipette up and down with a 10mL serological pipette to break up pellet as much as possible, and allow any cell clusters that fail to resuspend to settle to the bottom of the tube. Try not to carry these over to your new plate – remove any unbroken clusters that are transferred using a p100 pipette.

62 Aliquot cell suspension across plates (1 mL /well, one well at a time). Rock plate to distribute cells and incubate for 24:00:00 (37 °C ; 5% CO_2; 20% O_2). 1d

63 Following incubation, remove total volume of medium and replace with 2 mL /well N2:B27 **ONLY** (i.e. discontinue use of SMADi). Incubate plate (37 °C ; 5% CO_2; 20% O_2) for 24:00:00 . Continue replacing medium with fresh N2:B27 every 24 hours until **Day 12**. 1d

Differentiating PSCs to Neural Progenitors: Day 12 – np2

1d 0h 5m

64 Passage cells at Day 12, following **steps 52 - 63** and replating 1:1 in N2:B27 + [M] 10 micromolar (µM) ROCKi + [M] 200 micromolar (µM) AA2P. Continue replacing medium with fresh N2:B27 + [M] 200 micromolar (µM) AA2P every 24 hours until **Day 15/16**.

Differentiating PSCs to Neural Progenitors: Day 15/16 – np3

1d 0h 5m

65 Passage cells at Day 15/16, following **steps 52 - 63** and replating 1:1 in N2:B27 + 10 µM ROCKi + 200 µM AA2P.

> Note
>
> Passaging cells as clusters at Days 7 and 12 is critical for survival of the culture. Following Day 15/16 cells can and should be passaged as single cells, to promote proliferation and further differentiation. To this end, previous restrictions implemented in order to minimise fragmentation of the cell layer should now be removed. Use RT HBSS and accutase, and use a p1000 micropipette to fully dissociate cells. Cells may be resuspended using a 5mL serological pipette. From this point forward, centrifuge cells at 1250RPM for 2 minutes when passaging.

66 Continue replacing medium with fresh N2:B27 +AA2P every 24 hours until Day 18/19.

Differentiating PSCs to Neural Progenitors: Day 18/19 – np4

1d

67 Passage cells at Day 18/19, following **steps 52 - 63** and replating 1:1 in N2:B27 + [M] 10 micromolar (µM) ROCKi + [M] 200 micromolar (µM) AA2P.

68 Gently shake flasks to evenly distribute cells and incubate (37 °C ; 5% CO_2; 20% O_2) for 24:00:00 . 1d

Differentiating PSCs to Neural Progenitors: Day 19/20 1d 0h 5m

69 Following 24-hour incubation, remove total volume of media from each flask and replace with 15 mL /flask B27 + [M] 10 micromolar (µM) DAPT + [M] 200 micromolar (µM) AA2P.

Freezing NPCs 1d 0h 5m

70 Cool a Mr. Frosty to 4 °C .

71 Prepare freezing medium by adding 10% DMSO to N2:B27 + [M] 200 micromolar (µM) AA2P medium.

72 Aspirate the medium from the NPC culture and rinse each well with 1 mL of HBSS (without calcium and magnesium).

73 Add 1 mL of Accutase to each well containing cells. Swirl the dish gently to coat the entire surface.

74 Incubate the dish at 37 °C for 00:02:00 – 00:03:00 . When the cells begin to round up, remove the dish from the incubator. 3m

75 Prepare 15 mL tubes containing Room temperature DMEM/F12 at twice the volume of the Accutase to be added to the tubes.

76 Using a P1000 micropipette, gently lift the cells by pipetting up and down no more than five times.

77 Collect the cells in Accutase and transfer them directly into the prepared DMEM/F12.

78 Rinse the plates with 1 mL of DMEM/F12 per well and add the rinse to the tube containing Accutase, DMEM/F12, and cells.

79 Centrifuge the cell suspension at 1250 rpm, 00:02:00 . 2m

80 Label cryovials.

81 Carefully remove the supernatant and resuspend the cells in freezing medium, using 1 mL per well of a 6-well plate.

82 Transfer 1 mL of the suspension into each cryovial and place in a 4 °C Mr. Frosty. Transfer the cryovials to a -80°C freezer and leave for 24:00:00 . 1d

83 After 24 hours, transfer the cryovials to liquid nitrogen for long-term storage.

Thawing NPCs 1d 0h 2m

84 Prepare and prewarm the required volume of N2:B27 + [M] 200 micromolar (µM) AA2P medium containing [M] 10 micromolar (µM) ROCK inhibitor.

85 Prepare Geltrex-coated plates for one well of a 6-well plate per vial of cells.

86 Retrieve the required cryovials of NPCs from storage and place them on dry ice.

87 Rapidly thaw a vial of NPCs by placing it in a 37°C water bath until a small ice pellet remains.

88 Add 1 mL of prewarmed NPC medium to the cryovial and transfer the cell mixture to a 15 mL tube.

89 Centrifuge the cell suspension at 1250 rpm, 00:02:00 . 2m

90 Aspirate the supernatant and gently resuspend the pellet in N2:B27 + [M] 200 micromolar (µM) AA2P medium containing [M] 10 micromolar (µM) ROCK inhibitor.

91 Add 2 mL of the cell suspension per well of a 6-well plate using a P1000 micropipette or 100 µL per well of a 96-well plate using a P200 micropipette.

92 Ensure the cells are evenly spread in the well and incubate at 37 °C for 24:00:00 . 1d

Terminal Plating of Neural Progenitors: Coating Plates 12h

93 4h

Note

The following instructions are for plastic tissue culture-treated plates only. Plates must be coated 24 hours prior to terminal plating.

Coat plates with PDL 3 mL /flask (T25 flask) 1 mL /well (6w plate) 500 µL /well (12w plate) 300 µL /well (24w plate) 100 µL /well (96w plate) and incubate (37 °C ; 5% CO_2; 5% O_2) for at least 04:00:00 . Wash 3 times with 1 mL/well of DMEM or PBS.

94 Working On ice , dilute laminin stock (1 µL) to a concentration of 20 µL in cold DMEM.

95 Following incubation, aspirate total volume of DMEM or PBS solution from plates. Coat plates with laminin solution at the following volumes: 3 mL /flask (T25 flask) 8h

1 mL /well (6w plate) 500 µL /well (12w plate) 250 µL /well (24w plate) 100 µL /well (96w plate) and incubate (37°C; 5% CO_2; 5% O_2) Overnight .

Terminal Plating of Neural Progenitors: Terminal Plating

1d 0h 5m

96 Rinse wells to be passaged with 1 mL /well Room temperature HBSS.

97 Add Room temperature Accutase at 1 mL /well and incubate at 37 °C for 00:03:00 . While plates are incubating, prepare 15 mL tube containing Room temperature DMEM at twice the volume of the number of wells being passaged.

3m

98 Check that cells have detached by gently shaking plate. Using a P1000 micropipette, pipette up and across the well several times in order to create a homogenous, single-cell suspension. Transfer cells in Accutase to tube containing DMEM.

99 Centrifuge cells at 1250 rpm, 00:02:00 .

2m

100 Prepare plates by aspirating 1x DPBS and replacing with 1 mL /well (6w plates) 600 µL /well (12w plates) 300 µL /well (24w plates) 70 µL /well (96w plates) B27 + [M] 10 micromolar (µM) DAPT + [M] 200 micromolar (µM) AA2P + [M] 10 micromolar (µM) ROCKi.

101 Aspirate supernatant from atop pelleted cells. Resuspend in at least 1 mL B27 + [M] 10 Mass Percent DAPT + [M] 200 micromolar (µM) AA2P + [M] 10 micromolar (µM) ROCKi.

102 Using a P1000 micropipette, gently pass the cell suspension through a 40 µm cell strainer into a new tube to remove any remaining large colonies.

103 Collect 10 µL cell suspension and transfer to 0.6 mL Eppendorf tube. Add 10 µL trypan blue and mix by gently pipetting.

104 Use an automated cell counter or a hemocytometer to calculate the number of viable cells.

105 Calculate the volume of cell suspension containing the number of cells required to plate 200000 µL across all coated plates and transfer to a clean 50 mL tube.

106 Add a sufficient volume of B27 + [M] 10 micromolar (µM) DAPT + [M] 200 micromolar (µM) AA2P + [M] 10 micromolar (µM) ROCKi to cell suspension, such that the total volume will be enough to distribute cell suspension across all plates at the following volumes: 6 mL /flask (T25 flask) 1 mL /well (6w plate) 365 µL /well (12w plate) 200 µL /well (24w plate) 30 µL /well (96w plate).

107 Aliquot cell suspension across plates according to the volumes given in the previous. Gently shake plates to evenly distribute cells and incubate (37°C; 5% CO_2; 20% O_2) for 24:00:00 .

1d

108 Following 24-hour incubation, remove total volume of media and replace with 2 mL /well (6w plate) 750 µL /well (12w plate) 400 µL /well (24w plate) 100 µL /well (96w plate) B27 + [M] 10 micromolar (µM) DAPT + [M] 200 micromolar (µM) AA2P. Replace medium every 24 hours until **D7** following terminal plating.

109

Note

While cells should be treated with DAPT for 6 days following terminal plating, it is not essential to change medium every day. Specifics are left to the discretion of the operator.

On **D7** following terminal plating, remove the total volume of medium from cells and replace with 2 mL /well (6w plate) 750 µL /well (12w plate) 400 µL /well (24w plate) 100 µL /well (96w plate) B27 + [M] 200 micromolar (µM) AA2P.

110 Part media change (i.e. remove half the media and replace with an equal volume of fresh B27 + [M] 200 micromolar (µM) AA2P) every 7 days for the remaining time the cells are in culture.

111 After day 30 add 2 µL of laminin to the media for feeding cells every 7 days.