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Excitatory neuron differentiation from inducible Ngn2 iPSCs (WTC11)

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We use this protocol and it's working

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Disclaimer

This protocol was adapted from work in the Yin Shen lab and the Li Gan lab and used by Boxun Li in the Gersbach lab at Duke University.

Abstract

This protocol describes methods for generating excitatory neurons from a wild type male iPSC line (WTC11) containing doxycycline-inducible mouse neurogenin-2 (Ngn2) integrated at the AAVS1 safe-harbor locus.

Guidelines

All work must be performed in a sterile environment.

Materials

	A	B	C
	Reagent	**Catalog #**	**Vendor**
	ROCK Inhibitor Y-27632	72304	STEMCELL
	Corning‰ Matrigel‰ hESC-Qualified Matrix, LDEV-free	354277	Corning
	AccutaseTM Cell detachment solution	07922	STEMCELL
	MEM Non-Essential Amino Acids Solution (100X) (NEAA)	11140-050	Life Technologies
	GlutaMAX ‰ Supplement (100X)	35050-061	Life Technologies
	Recombinant Human/Murine/Rat BDNF	450-02	PeproTech
	Recombinant Human NT-3	450-03	PeproTech
	Laminin Mouse Protein, Natural	23017-015	Life Technologies
	Doxycycline hyclate	D9891	Sigma-Aldrich
	B-27‰ Supplement (50X)	17504-044	Life Technologies

	A	B	C
	N-2 Supplement (100X)	17502-048	Life Technologies
	DMEM/F-12, HEPES	11330-032	Life Technologies
	KnockOut ‰ DMEM/F-12	12660-012	Life Technologies
	Neurobasal ‰-A Medium	12349-015	Life Technologies
	DPBS, no calcium, no magnesium	14190-144	Life Technologies
	Poly-D-Lysine	P6407	Sigma-Aldrich

Differentiation protocol

1 Differentiation media: Prepare the pre-differentiation medium and maturation medium according to the table below. BDNF, NT-3, laminin, and doxycycline should be added immediately before use.

	A	B	C	D
	Medium	**Reagents**	**FinalConcentration**	**Example(100 mL)**
	Pre-differentiation Medium	KnockOut‰ DMEM/F-12	100%	98 mL
	Pre-differentiation Medium	N-2 Supplement (100X)	1X	1 mL
	Pre-differentiation Medium	NEAA (100X)	1X	1 mL
	Pre-differentiation Medium	BDNF	10 ng/mL	Add before use
	Pre-differentiation Medium	NT-3	10 ng/mL	Add before use
	Pre-differentiation Medium	Laminin	1 µg/mL	Add before use
	Pre-differentiation Medium	Doxycycline	2 µg/mL	Add before use
	Maturation Medium	Neurobasal‰-A Medium	50%	48.5 mL
	Maturation Medium	DMEM/F-12, HEPES	50%	48.5 mL
	Maturation Medium	NEAA (100X)	1X	1 mL
	Maturation Medium	B-27‰ Supplement (50X)	0.5X	1 mL

	A	B	C	D
	Maturation Medium	N-2 Supplement (100X)	0.5X	0.5 mL
	Maturation Medium	GlutaMAX ‰ Supplement (100X)	0.5X	0.5 mL
	Maturation Medium	BDNF	10 ng/mL	Add before use
	Maturation Medium	NT-3	10 ng/mL	Add before use
	Maturation Medium	Laminin	1 µg/mL	Add before use

2 Pre-differentiation (Day -3 to Day 0)

2.1 Day -4:

(1) Use Accutase to dissociate the WTC11 inducible Ngn2 iPSCs following the Gladstone Stem Cell Core iPSC Maintenance protocol.
(2) Seed the cells in Matrigel-coated plates using mTeSR plus medium with 5 µM ROCK Inhibitor. Use the following table as a guideline:

	A	B	C
	Plate Type	**Media Volume**	**Cell Density**
	12-well plate	1 mL / well	500K / well
	6-well plate	2 mL / well	1M / well
	10-cm dish	10 mL / dish	5M / dish

(3) Optional: Transduce cells with lentivirus (by adding the appropriate amount for target MOI to the cells) immediately after plating. Rock plate to mix well. Lentivirus can also be introduced later.

2.2 Day -3:

Change media using pre-differentiation medium without ROCK inhibitor.

2.3 Day -2:

Change media using pre-differentiation medium without ROCK inhibitor.

2.4 Day -1:
• Change media using pre-differentiation medium without ROCK inhibitor.
• Coat new plates for differentiation using 10 µg/mL Poly-D-Lysine in molecular biology-grade water, incubating at 37°C overnight.

3 Post-differentiation (Day 0+)

3.1 Day 0:
(1) Wash the Poly-D-Lysine-coated plates three times using DPBS. Air dry in a tissue culture hood until all the DPBS is evaporated.
(2) Dissociate the pre-differentiated cells using Accutase. For example, using 6-well plates:
(2.1) Add 0.5 mL Accutase to each well and incubate at 37°C until cells are dissociated into single cells (~5 minutes).
(2.2) Add DPBS and collect the cells (do not pipette at this stage).
o First, add 1 mL DPBS and collect the cells without pipetting.
o Add 1.5 mL DPBS to rinse the wells and collect all the cells.
(2.3) Centrifuge for 5 minutes at 200 RCF at room temperature.
(2.4) Aspirate the supernatant.
(2.5) Resuspend in 1 mL Maturation Media and use a P1000 pipette to dissociate the pellet into single cells. Triturate at least 30X.
(2.6) Count the number of live cells using Trypan blue. At least 90% of the cells should be single cells.
(3) Plate the cells using Maturation Medium with 2 µg/mL doxycycline using the following table:

	A	B	C
	Plate Type	**Media Volume**	**Cell Density**
	24-well plate	0.5 mL / well	250K / well
	12-well plate	1 mL / well	500K / well
	6-well plate	2 mL / well	1M / well
	10-cm dish	10 mL / dish	5M / dish
	15-cm dish	25 mL / dish	12.5M / dish

(4) Optional: Transduce cells with lentivirus (by adding the appropriate amount of target MOI to the cells) immediately after plating. Rock plate to mix well.
(5) Optional: Activate dCas9-VPH by adding 20uM trimethoprim (TMP) to the medium after plating. Rock plate to mix well. TMP can also be included or excluded during media changes later depending on experimental design.

3.2 Day 1:
Optional: Add mouse astrocytes prepared from P1 C57BL/6 mouse cerebral cortices (that have been cultured in astrocyte growth medium in vitro for 7-8 days) at a ratio of 5 neurons to 1 astrocyte. The astrocytes are dissociated with Trypsin-EDTA and resuspended in neuronal maturation medium (listed at the start of this protocol) to 500K/mL. The added volume should be 1/5 of the existing medium.

3.3 Day 7:
Remove half the volume of media from each well or dish and add the same amount of fresh Maturation Medium without doxycycline.

3.4 Day 14:
• Remove half the volume of media from each well or dish and add twice the amount of fresh Maturation Medium without doxycycline.
• For example, using 12-well plates, first remove 0.5 mL then add 1 mL for a total of 1.5 mL.

3.5 Day 21:
• Remove 1/3 of the media and replace twice the amount of fresh Maturation Medium without doxycycline.
• At this point, the total volume of Maturation Medium should be double the initial volume. For example, using 12-well plates, there should be 2 mL total.

3.6 After Day 21:
• Change 1/3 of the media using fresh Maturation Medium without doxycycline every week.