iNeuron

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iNeuron pre-differentiation & differentiation protocol V.2

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1 Works for me dx.doi.org/10.17504/protocols.io.bcrjiv4n

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ABSTRACT

This protocol describes thedifferentiation of iPSCs with stably integrated doxycycline-inducible Ngn2 (such as i3Ns).

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Tian et al (2019). CRISPR Interference-Based Platform for Multimodal Genetic Screens in Human iPSC-Derived Neurons. Neuron pii: S0896-6273(19)30640-3. [Epub ahead of print] PubMed PMID: 31422865.

ATTACHMENTS

iNeuron pre-differentiation & differentiation protocol (1).pdf

MATERIALS

NAME	CATALOG #	VENDOR
Glutamax (100x)	35050-061	Gibco - Thermo Fischer
DPBS no calcium no magnesium	14190250	Gibco - Thermo Fischer
KnockOut™ DMEM	10829018	Thermo Fisher
MEM Non-Essential Amino Acids Solution (100X)	11140050	Thermo Fisher
DMEM/F-12	11320033	Thermo Fisher
B-27™ Supplement (50X), minus vitamin A	12587010	Thermo Fisher
KnockOut™ DMEM/F-12	12660012	Thermo Fisher
N-2 Supplement (100X)	17502048	Thermo Fisher
Laminin Mouse Protein, Natural	23017015	Thermo Fisher
StemPro™ Accutase™ Cell Dissociation Reagent	A1110501	Thermo Fisher
Essential 8™ Medium	A1517001	Thermo Fisher
Rock inhibitor Y-27632 dihydrochloride	125410	Tocris
Neurobasal™-A Medium	10888022	Thermo Scientific
BrainPhys™ Neuronal Medium	05790	Stem Cell Technologies
Recombinant Human/Murine/Rat BDNF	450-02	peprotech
Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix	356231	Corning
Recombinant Human NT-3	450-03	peprotech
Doxycycline hydrochloride	D3447	Sigma – Aldrich

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Culture iPSCs

1

WTc11 iPSC Culture and Maintenance
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PREVIEW RUN

1.1 Thaw the frozen StemFlex Supplement 10X at **Room temperature** for ~ **02:00:00** or overnight at **2 °C** to **8 °C** .

IMPORTANT! Do not thaw the frozen supplement at 37°C.

1.2

Mix the thawed supplement by gently inverting 3–5 times.

1.3 Aseptically transfer 50 mL of StemFlex Supplement 10X to the bottle of StemFlexTM Basal Medium (450 mL fill).

1.4

Gently invert the bottle several times to obtain 500 mL of homogenous complete medium.

Following reconstitution, complete StemFlexTM Medium can be stored at 2°C to 8°C for up to 2 weeks or aliquoted and stored at **-5 °C** to **-20 °C** for up to 6 months. Alternatively, usage size aliquots of the supplement can be made and frozen at **-5 °C** to **-20 °C** for up to 6 months. Avoid multiple freeze-thaw cycles.

1.5 Feed the PSCs the day after seeding followed by every-other-day thereafter.

If the cells are to be left without feeding for two days (for example, over a weekend), then double the feed volume (i.e., 4 mL added per well of 6-well plate).

1.6 iPSCs should be split when cells are ~ 80% confluent.

1.7 Thaw Matrigel on ice and dilute in pre-chilled Knockout DMEM for a final volume of **100 Mass Percent** .

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1.8

Coat desired wells/plates with diluted Matrigel and incubate at **37 °C** for **00:30:00** - **01:00:00** using the following table for volumes to add **per well**:

Per:	96-well	24-well	12-well	6-well	10-cm dish	15-cm dish
Volume to add:	40 µL	200 µL	0.5 mL	1 mL	5 mL	10 mL

Matrigel may be re-used during this time only to coat additional plates. Original plates should have PBS or media to prevent the matrix from drying out. Matrigel coated plates must be used within 14 days of coating.

1.9 Tilt cell-containing plate towards you and aspirate existing media.

1.10

Wash wells once with ample PBS (about 2x amount of media).

1.11

Add accutase to well(s) using the following table for volumes per well and incubate at **37 °C** for **00:03:00** ; add another **01:00:00** - **00:02:00** if cells have not mostly lifted/dissociated.

Per:	96-well	24-well	12-well	6-well	10-cm dish	15-cm dish
Volume to add:	20 µL	100 µL	250 µL	0.5 mL	2 mL	4 mL

1.12 Add ample PBS to accutase-containing well(s) to dilute accutase using the following table for volumes per well:

Per:	96-well	24-well	12-well	6-well	10-cm dish	15-cm dish
Volume to add:	200 µL	1 mL	2.5 mL	5 mL	10 mL	20 mL

1.13

Pipette up and down gently to mechanically release remaining cells, collect, and add to appropriately-sized conical tubes.

1.14

Spin cells at **200 x g** for **00:05:00** at **Room temperature** .

1.15 Carefully aspirate supernatant from pelleted conicals.

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1.16 Add appropriate volume of StemFlex + Rock inhibitor at [M]**10 Micromolar (µM)** to conicals according to pellet size for counting.

Rock inhibitor should only be used when cells are individualized or in small colonies (typically the first two days after passaging); the presence of Ri at higher densities results in cell stress/death, and in general, Rock inhibitor greatly reduces proliferation.

For first time use of Rock inhibitor, it is suggested to aliquot at 10mM [1000x], diluting in DPBS, and use on cells at concentration [M]**10 Mass Percent** .

1.17 

Triturate to resuspend cells in StemFlex + Rock inhibitor and remove 10µL and add this volume to a 1.5mL Eppendorf tube.

Be careful to minimize contact of pipette with the side of the conical wall.

1.18 Count cells and calculate desired number of cells to seed, and dilute this volume with additional StemFlex + Rock inhibitor to plate using the following table for volume to add per well:

Per:	96-well	24-well	12-well	6-well	10-cm dish	15-cm dish
Volume to add:	50-100 µL	250-500 µL	0.75-1 mL	1.5-2 mL	8-12 mL	15-25 mL

For general passaging where exact cell number seeded is not important, adding resuspended cells at 1:100, 1:50, and 1:20 the final well volume typically provides near-confluency in 7 days, 5 days, and 3 days, respectively.

1.19 

iPSCs can be frozen in StemFlex + 10% DMSO.

Days -3-0: Pre-differentiation

2

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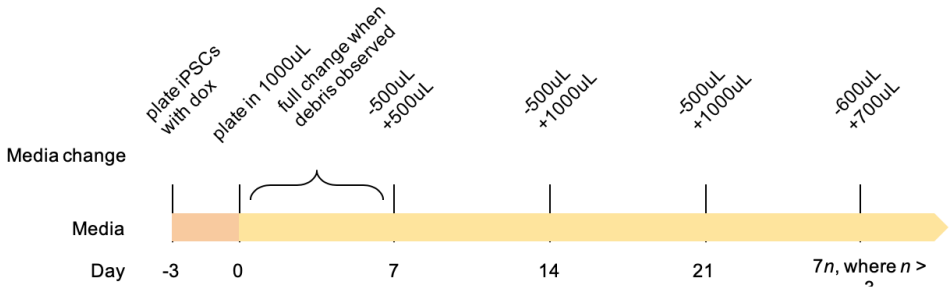
Pre-Differentiation and Differentiation Notes:

1. Keep reagents at 4 °C for a maximum of 4 weeks, If needing long term storage, don't freeze-thaw more than 3 times.

2. Sometimes pre-differentiated cells take a long time to come off of the plate on Day 0. This is usually okay. It is important that your cells are single cells.

3. Feeding schedule and volumes:



Make N2 Pre-differentiation Media:

Component	Stock Concentration	Final Concentration	Dilution Factor	For 100 mL (mL)	For 50 mL (units in cell)
Konckout DMEM/F12	1X	1X	1	100	50mL
NEAA	100X	1X	100	1	500uL
N2 Supplement	100X	1X	100	1	500uL
NT-3	10ug/mL	10ng/mL	1000	0.1	50uL
BDNF	10ug/mL	10ng/mL	1000	0.1	50uL
Mouse Laminin*	*1.0mg/mL*	*1ug/mL*	*1000*	*0.1*	*50uL*
ROCK Inhibitor**	*10mM*	*10uM*	*1000*	*0.1*	*50uL*
Doxycycline***	*2mg/mL*	*2ug/mL*	*1000*	*0.1*	*50uL*

*Mouse Laminin comes at variable concentrations, so make sure the final concentration is correct in the media you are making!
**Only add during plating of iPSCs on Day -3 and omit for any further media changes
***Add immediately before use

3 Coat plate with matrigel diluted in Knockout DMEM. Coat for at least 00:30:00 (or O/N). Coated plates can last in the 37 °C incubator for 14 days.

4 Aspirate media from iPSCs and wash with DPBS.

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5 Add Accutase and incubate at **37 °C** for **00:03:00** ; if necessary, incubate for additional time up to **00:07:00** .

6 Use gentle agitation to release cells, and collect in an Eppendorf tube or conical with DPBS.

7 Spin cells at **200 x g** for **00:05:00** resuspend in N2 pre-diff media.

8 Count cells and add desired amount to an Eppendorf tube or conical, spin, resuspend in N2 pre-differentiation media, and plate onto Matrigel-coated cell culture vessels for the pre-differentiation. Reference seeding density chart for plating

9 Perform ½ pre-differentiation media change every day (w/dox, no RI) throughout days 0-3 pre-differentiation period or full media change on day -1 or day -2. **Be consistent.**

10 Going into differentiation, it is best if cells are in single-cell suspension. Optional: use cell strainer.

11 Pre-differentiated cells can be frozen in 10%DMSO + N2/B27 Differentiation media (**can also freeze in pre-diff media).

Day 0: Releasing and Plating Pre-Differentiated iNeurons

12 Make N2/B27 Differentiation Media:

Component	Stock Concentration	Final Concentration	Dilution Factor	For 100mL (mL)	For 50 mL (units in cell)
DMEM/F12*	*1X*	*0.5X*	*2*	*50*	*25mL*
Neurobasal-A	1X	0.5X	2	50	25mL
NEAA	100X	1X	100	1	500uL
GlutaMAX	100X	0.5X	200	0.5	250uL
N2 Supplement	100X	0.5X	200	0.5	250uL
B27-VA Supplement	50X	0.5X	100	1	500uL
NT-3	10ug/mL	10ng/mL	1000	0.1	50uL
BDNF	10ug/mL	10ng/mL	1000	0.1	50uL
Mouse Laminin**	*1.0mg/mL*	*1ug/mL*	*1000*	*0.1*	*50uL*
Doxycycline***	*2mg/mL*	*2ug/mL*	*1000*	*0.1*	*50uL*

*DMEM/F12 with either bicarbonate or HEPES buffer is okay, but we have had qualitatively better neurons with HEPES buffer when grown and differentiated side-by-side. HEPES is essential for imaging, as media will change color after ~30 minutes if using bicarbonate buffered media.
**Mouse Laminin comes at variable concentrations, so make sure the final concentration is correct in the media you are making!
***Add immediately before use and only on Day 0

13 Aspirate media and wash with DPBS.

14 Add Accutase and incubate at **37 °C** for **00:03:00** ; if necessary, incubate for additional time up to **00:07:00** .

15 Use gentle agitation to release cells, and collect in an Eppendorf tube or conical with DPBS.

16 Spin cells at **200 x g** for **00:05:00** .

17 Carefully aspirate DPBS/accutase solution and resuspend in Classic N2/B27 Differentiation Media.

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18 Count cells, dilute to appropriate density, and plate onto PDL-coated cell culture vessels.

19 Leftover cells can be frozen down in pre-differentiation media + 10% DMSO.

Day 0+: Neuronal differentiation

20 Full media change on day 3 post-plating, once debris is observed.

21 Pipet at the wall of the plate. **DO NOT TOUCH BOTTOM OF PLATE!** Be extra careful with 96 well plates.

22 Change Classic N2/B27 Differentiation media at minimum once every week without dox or rock inhibitor.

23 Closely monitor. Wait for a week at minimum before using for experiment. Preferably, use after 2 weeks.

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