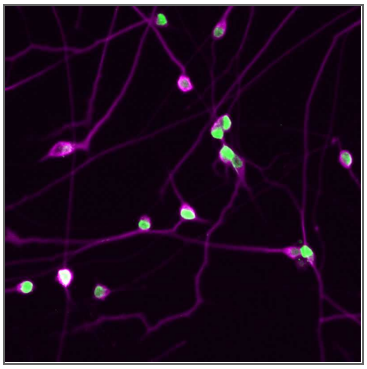

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Differentiation of RGC Induced Neurons (RGC-iNs) V.2

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ABSTRACT

This protocol is designed to convert human induced pluripotent stem cells (PSCs) into retinal ganglion cell induced neurons (RGC-iNs) using a doxycycline-inducible polycistronic transcription factor gene cassette containing human NEUROG2, ATOH7, ISL1, and POU4F2. The TetO-driven transgene cassette is integrated into the CLYBL safe harbor site using a CRISPR-Cas12a ribonucleoprotein. The process of generating neurons is greatly enhanced by the inclusion of the BMP blocker LDN-193189.

GUIDELINES

Apart from observation under the microscope, counting, and centrifugation, all steps should be carried out in a sterile biological safety cabinet.

MATERIALS

Table: Key resources or reagents required.

A	B	C
REAGENT or RESOURCE	SOURCE	IDENTIFIER
40µm Cell Strainer, EASYstrainer (for generating single cell suspension during plating)	Greiner	Cat# 542040
Accutase (single cell passaging of hPSCs)	Sigma-Aldrich	Cat# A6964
B27 vitamin (neural supplement)	Thermo Fisher Scientific	Cat# 17504044

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Protocol status: Working
We use this protocol and it's working

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PROTOCOL integer ID: 88422

	A	B	C
	BDNF (growth factor for RGC growth and survival)	Qkine	Cat# Qk050
	Blebbistatin (ROCK inhibitor for improving cell survival)	Sigma-Aldrich	Cat# B0560
	BrainPhys Neuronal Medium (basal media for supporting long-term growth of neurons)	StemCell Technologies	Cat# 05790
	CultureOne supplement (for enhancing neural conversion)	Thermo Fisher Scientific	Cat# A3320201
	DMEM (basal media)	Thermo Fisher Scientific	Cat# 11965
	DMEM/F12 50:50 (basal media)	Thermo Fisher Scientific	Cat# 11330
	Doxycycline hyclate (antibiotic for transgene induction)	Sigma-Aldrich	Cat# D5207
	F12 (basal media)	Thermo Fisher Scientific	Cat# 11765
	GDNF (growth factor for enhancing RGC growth and neuronal survival)	Qkine	Cat# Qk051
	Insulin-Human Recombinant (N2 supplement component)	Roche	Cat# 11376497001
	L-ascorbic acid (N2 supplement component)	Sigma-Aldrich	Cat# A8960
	LDN-193189 (pre-patterning BMP pathway inhibitor)	Sigma-Aldrich	Cat# SML0559
	Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix (cell attachment/differentiation of hPSCs)	Corning	Cat# 354230
	mTeSR1 (maintenance and propagation of hPSCs)	Stem Cell Technologies	Cat# 85850
	N-2 Supplement (neural supplement)	Thermo Fisher Scientific	Cat# 17502048
	NEAA (non-essential amino acids for supporting neuronal growth)	Thermo Fisher Scientific	Cat# 11140
	Nicotinamide (NIC) (vitamin B3 supplement to enhance differentiation)	Sigma-Aldrich	Cat# 72340
	Poly-L-ornithine (PLO) hydrobromide (for neural attachment)	Sigma Aldrich	Cat# P3655
	Progesterone (N2 supplement component)	Sigma-Aldrich	Cat# P8783
	Putrescine dihydrochloride (N2 supplement component)	Sigma-Aldrich	Cat# P5780
	Sodium selenite (N2 supplement component)	Sigma-Aldrich	Cat# S5261
	Thiazovivin (alternate ROCK inhibitor for cell survival)	LC Labs	Cat# T-9753
	holo Transferrin Human (N2 supplement component)	Sigma-Aldrich	Cat# T0665

	A	B	C
	GlutaMAX Supplement (auxiliary energy source for cells)	Thermo Fisher Scientific	Cat# 35050061

BEFORE START INSTRUCTIONS

For media/reagent recipes see the last section of the protocol.

PSC expansion step

1 Grow PSCs in mTeSR1 under hypoxia ([M] 5 % (v/v) O_2/ [M] 10 % (v/v) CO_2) or normoxia ([M] 20 % (v/v) O_2/ [M] 5 % (v/v) CO_2) at 37 °C .

12-well plate (3.5 cm^2): Plate 5,000 PSCs into each well of 12-well plates in mTeSR1 in the presence of [M] 5 micromolar (µM) blebbistatin (blebb; 2,000x stock). Feed daily in mTeSR1 (without blebb) for ~4 more days. Typically, we get ~200,000 – 500,000 cells per well when cells are ready for passaging.

6-well plate (9.6 cm^2): Plate 15,000 PSCs into each well of 6-well plates in mTeSR1 in the presence of blebb. Feed daily in mTeSR1 (without blebb) and grow for ~4 more days. Typically, we can get ~750,000 – 1,000,000 cells per well when cells are ready for passaging.

Note

For PSC expansion make sure that colonies are not overgrown (70-80 % confluent) and not touching.

Alternatively, [M] 2 micromolar (µM) thiazovivin (10 mM or 5000x stock) can be used instead of blebb as a ROCK inhibitor.

Day -1: Priming of stem cells for neural induction

2 1d One day prior to neural induction, pre-coat TC plates overnight with [M] 0.1 mg/mL poly-L-ornithine hydrobromide (PLO). To do this, add 1 mL [M] 2 mg/mL PLO (20x) to 19 mL cell culture grade H2O, use 1 mL per well for 6-well plates, 0.5 mL per well for 12 well plates and 0.25 mL per well for 24 well plates. Incubate overnight at 37 °C .

Pre-treat stem cells by replacing mTeSR1 media with fresh mTeSR1 supplemented with

[M] 1 µg/mL doxycycline (dox; 1,000x stock) and [M] 100 nanomolar (nM) LDN-193189 (10,000x stock).

Note

For long-term experiments, you need better adhesion of cells so you can use a higher concentration of PLO. To do this, dilute 1 mL [M] 2 mg/mL PLO (20x) into 9 mL cell culture grade H2O for a final concentration of [M] 0.2 mg/mL.

Day 0: Neural Induction

3 Wash overnight PLO-coated plates >3 times with culture grade H2O. Let plates dry completely in the back of the TC hood for 01:00:00, then coat with [M] 1 % (v/v) Matrigel. For Matrigel coating, use 1 mL per well for 6-well plates, 0.5 mL per well for 12 well plates and 0.25 mL per well for 24 well plates. Incubate for > 03:00:00 at 37 °C. 4h

4 Prepare Neural Induction Medium (NIM) initiation cocktail with [M] 2 µg/mL dox (500x stock), [M] 100 nanomolar (nM) LDN (10,000x stock), 1x CultureOne (100x stock) and [M] 5 micromolar (µM) blebb.

Note

Dox is light-sensitive, so keep aliquots cool after thawing and dark when not in use (up to ~1 month at 4 °C).

In addition, it is critical to prevent the drying of matrigel-coated dishes after the matrigel has been added.

5 Incubate the cells with prewarmed Accutase (~ 00:05:00) in the incubator for 00:12:00 The volume of Accutase to use is 1/2 the volume that you maintain the cells in (e.g., 1 mL per well of a 6 well plate, 0.5 mL per well of a 12 well plate and 0.25 mL per well of a 24 well plate). 17m

6 Gently rinse the wells using a P1000 micropipette and pipet up and down 3 times to further break up the cell clumps into single cells.

7 Put the cells into a conical 1.5 mL or 5 mL tube with 2 times the volume of prewarmed mTeSR1 with [M] 5 micromolar (µM) blebb (e.g. 1 mL Accutase + 2 mL mTeSR1) to quench the Accutase, then pellet the cells for 00:05:00 at 80 x g. 5m

8 Aspirate the supernatant and then resuspend the cell pellet in prewarmed NIM + [M] 5 micromolar (µM) blebb. 2m

9 Filter cells with a 40 µm cell strainer. Count the cells with a hemocytometer. 5m

10 Plate cells onto PLO/matrigel-coated plates. 5m

To make a 6-well plate **(9.6 cm^2/well; 57.6 cm^2 total; 7,000 cells/cm^2)**: Add 403,200 cells into 12 mL (67,200 cells per well) of NIM initiation cocktail (NIM + dox, LDN, CultureOne, blebb) in a 15 mL conical tube, mix well and distribute across the wells (2 mL per well).

To make a 12-well plate **(3.5 cm^2/well; 42 cm^2 total; 7,000 cells/cm^2)**: Add 294,000 cells into 12 mL (24,500 cells per well) of NIM initiation cocktail in a 15 mL conical tube, mix well and distribute across the wells (1 mL per well).

To make a 24-well plate **(1.9 cm^2/well; 45.6 cm^2 total; 7,000 cells/cm^2):** Add 319,200 cells into 12 mL (13,300 cells per well) of NIM initiation cocktail in a 15 mL conical tube, mix well and distribute across the wells (0.5 mL per well).

Day 1: Maintenance

11 Do nothing.

Day 2: Feed – add 1/3 of media

12 For 6 well plate: Add 1 mL **NIM +** [M] 1 µg/mL **dox + 1xCultureOne** to each well. 5m

For 12 well plate: Add 0.5 mL **NIM + dox + CultureOne** to each well.

For 24 well plate: Add 0.25 mL **NIM + dox + CultureOne** to each well.

Note

Do this very carefully by adding media to the sides of the dish. If you are not very careful cells will detach.

Day 3: Maintenance

13 Do nothing.

Day 4: Feed – exchange 1/3 of media

14 For 6 well plate: Remove 1 mL media and replace with fresh 1 mL **NIM +** [M] 1 µg/mL **dox + 1xCultureOne + NIC (** [M] 10 millimolar (mM) **).** 5m

For 12 well plate: Remove 0.5 mL media and replace with fresh 0.5 mL **NIM + dox + CultureOne + NIC.**

For 24 well plate: Remove 0.25 mL media and replace with fresh 0.25 mL **NIM + dox + CultureOne + NIC.**

Beyond day 4, plates need to be fed every other day.

Note

This is the last day to add CultureOne.

For all subsequent exchanges:
Neurons tend to easily dissociate from the dish, so be very careful when aspirating. Take care to aspirate and dissociate by tilting the dish so that the medium accumulates on one side. Then, aspirate/dispense with the pipette directed toward the wall of the dish (i.e., away from the cells at the bottom).

Day 6: Feed – exchange 1/3 of media

15 For 6 well plate: Remove 1 mL media and replace with fresh 1 mL **BrainPhys + B27 (1x) + [M] 1 µg/mL dox + BDNF ([M] 50 ng/mL) + GDNF ([M] 10 ng/mL) + NIC ([M] 10 millimolar (mM)).** 5m

For 12 well plate: Remove 0.5 mL media and replace with fresh 0.5 mL **BrainPhys + B27 + dox + BDNF + GDNF + NIC.**

For 24 well plate: Remove 0.25 mL media and replace with fresh 0.25 mL **BrainPhys + B27 + dox + BDNF + GDNF + NIC.**

> Note
>
> This is the last day to add dox.

Day 8: Feed – exchange 1/3 of media

16 For 6 well plate: Remove 1 mL media and replace with fresh 1 1 mL **BrainPhys + B27 (1x) + BDNF ([M] 50 ng/mL) + GDNF ([M] 10 ng/mL) + NIC ([M] 10 millimolar (mM)).** 5m

For 12 well plate: Remove 0.5 mL media and replace with fresh 0.5 mL **BrainPhys + B27 + BDNF + GDNF + NIC.**

For 24 well plate: Remove 0.25 mL media and replace with fresh 0.25 mL **BrainPhys + B27 + BDNF + GDNF + NIC.**

17 For long-term experiments >1 week, continue feeding by 1/3 media exchange every other day with **BrainPhys + B27 + BDNF + GDNF + NIC**. 5m

Media/Reagent Recipes

18 **Neural Induction medium (NIM medium):**

	A	B	C
	Component	**Catalog #**	**Volume**
	DMEM/F12, HEPES	Thermo Fisher Scientific # 11330	485 ml
	N2 supplement (100x)	Thermo Fisher Scientific # 17502048 or recipe below	5 ml

	A	B	C
	NEAA (non-essential amino acids, 100x)	Thermo Fisher Scientific # 11140	5 ml
	GlutaMAX supplement (100x)	Thermo Fisher Scientific # 35050061	5 ml
			Total 500 ml

19 **N2 supplement (100X) recipe -** If making in-house add the following:

	A	B	C	D	E	F	G
	Component	**Catalog #**	**100 ml**	**150 ml**	**200 ml**	**250 ml**	**500 ml**
	Transferrin	Sigma-Aldrich # T0665	1 g	1.5 g	2 g	2.5 g	5.0 g
	Insulin	Roche # 11376497001	50 mg	75 mg	100 mg	125 mg	250 mg
	Progesterone	Sigma-Aldrich # P8783	63 µg	94.5 µg	126 µg	157 µg	315 µg
	Putrescine	Sigma-Aldrich # P5780	161 mg	241.5 mg	322 mg	402.5 mg	805 mg
	Sodium Selenite	Sigma-Aldrich # S5261	50.2 µg	75.3 µg	100.4 µg	125.5 µg	251 µg
	DMEM/F12	Thermo Fisher Scientific # 11330	**to 100 ml**	**to 150 ml**	**to 200 ml**	**to 250 ml**	**to 500 ml**

20 **Reagent Stock Dilutions:**

Recombinant Human BDNF Protein (Qkine, Cat# Qk050): [M] 50 µg/mL stock in [M] 10 millimolar (mM) HCl with [M] 0.1 % (v/v) BSA; 1,000X; use at [M] 50 ng/mL. Store aliquots in -80 °C.

Recombinant Human GDNF Protein (Qkine, Cat# Qk051): [M] 10 µg/mL stock in cell culture grade H2O with [M] 0.1 % (v/v) BSA; 1,000X; use at [M] 10 ng/mL. Store aliquots in -80 °C.

Doxycycline (dox) (Sigma-Aldrich, Cat# D5207): Make [M] 1 mg/mL stock (working concentration is [M] 0.5-2 µg/mL) in cell culture grade ddH2O and filter sterilize; 1,000X; use at [M] 1 µg/mL. Store 1 mL aliquots at -20 °C. Protect from light.

LDN-193189 hydrochloride (LDN) (Sigma Cat# SML0559-5MG): [M] 1 millimolar (mM) (10,000x):

g = Molecular Weight (g/mol) * Molarity (M) * Volume (L)
0.005 g = (406.48 g/mol) * (0.001 M) * (Volume)
0.005 g = 0.41 g/L * Volume
Volume = 0.012 L or 5 mg LDN in 12 mL DMSO; Store aliquots in -80 °C.

Matrigel (GF reduced) ([M] 1 % (v/v) **)** (Corning, Cat# 354230):
Thaw stock 10ml bottle overnight On ice (wet) before aliquoting. Always keep matrigel and tubes on ice and never let it come to room temperature or it will gel.
Make 200 µL aliquots. Store aliquots in -80 °C.
Add 200 µL matrigel to 24 mL ice-cold DMEM/F12 (~ [M] 1 % (v/v) final).
Add 1 mL per well of 6-well plate, 0.5 mL per well of 12-well plate or 0.25 mL per well of 24-well plate.

Nicotinamide (NIC) (Sigma Cat# 72340): [M] 1 Molarity (M) (100x) stock solution ([M] 10 millimolar (mM) working solution). Soluble in water to ~ 1g/10ml.
g = Molecular Weight (g/mol) * Molarity (M) * Volume (L); g = (122.12g/mol)*(1M)*(0.05L) = 6.11 g NIC in 50 mL of DMEM/F12 (or water); filter sterilize and store at 4 °C.

poly-L-ornithine hydrobromide (PLO) - (Sigma Cat# P3655-500MG; mol wt 30,000-70,000 Da): [M] 2 mg/mL (20x) stock:
Add 500 mg PLO to 250 mL cell culture ddH2O.
Make 1 mL aliquots. Store aliquots in -80 °C.

Blebbistatin (blebb) ([M] 10 millimolar (mM) or 2,000x stock, Sigma Cat# B0560-1MG):
g = Molecular Weight (g/mol) * Molarity (M) * Volume (L)
0.001 g = (292.33 g/mol) * (0.01 M) * (Volume)
0.001 g = 2.92 g/L * Volume
Volume = 0.000342 L = 0.342 mL = 342 µL
Dissolve 1 mg blebbistatin into 342 µL DMSO, divide into 20 µL aliquots (store at -80 °C). Working concentration is [M] 5 micromolar (µM).