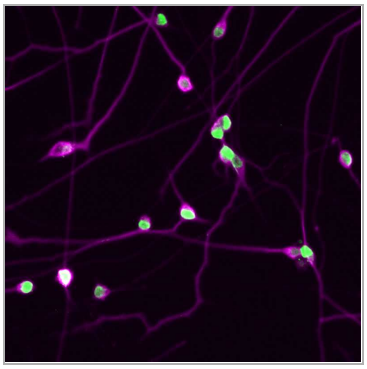

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Differentiation of RGC Induced Neurons (RGC-iNs)

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ABSTRACT

This protocol is designed to create induced retinal ganglion cells neurons (RGC-iNs) using a doxycycline-inducible polycistronic transcription factor cassette containing NEUROG2, ATOH7, ISL1, and POU4F2 and TetO integrated at the CLYBL safe harbor site. The cassette is integrated into the CLYBL safe harbor site using a CRISPR-Cas12a ribonucleoprotein. This process of generating neurons is greatly enhanced by the inclusion of the BMP blocker LDN193189.

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
	All-trans retinoic acid (ATRA) (for enhancing cell survival)	Sigma-Aldrich	Cat# R2625
	Accutase (single cell passaging of hPSCs)	Sigma-Aldrich	Cat# A6964
	B27 vitamin A (-) (neural supplement)	Thermo Fisher Scientific	Cat# 12587010
	B27 vitamin (neural supplement)	Thermo Fisher Scientific	Cat# 17504044
	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

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

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PROTOCOL integer ID: 82827

	A	B	C
	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
	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
	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 iPSCs in 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 iPSCs into each well of 12 well plates in the presence of blebbistatin. Feed daily in mTeSR1 and grow for ~4 more days. Typically, we can get 200,00 –

500,000 cells per well when cells are ready for passaging.

6-well plate (9.6 cm^2): Plate 15,000 iPSCs into each well of 6-well plates in the presence of blebbistatin. Feed daily in mTeSR1 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.

Day -1: Priming of stem cells for neural induction

2 One day prior to neural induction, pre-coat TC plates overnight with [M] 0.1 mg/mL poly-L-ornithine (PLO). Add 1 mL 20x PLO to 19 mL cell culture grade H2O, use 1 mL per well of 1x PLO for 6-well plates (0.5 mL per well for 12 well plates) and incubate coated plate overnight at 37 °C .

Note

For long-term experiments, you need better adhesion of cells so you can dilute 1 mL 20x PLO 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 in the back of the TC hood for 01:00:00 , then coat with [M] 1 % (v/v) Matrigel (recommended > 03:00:00). 4h

4 Prepare Neural Induction Medium Initiation Cocktail (NIM) with [M] 2 µg/mL doxycycline (500x stock), [M] 100 nanomolar (nM) LDN, 1x CultureOne (100x stock) and [M] 5 micromolar (µM) blebbistatin (blebb; 2,000x stock). Place in 37 °C bead bath to warm during the dissociation process.

Note

Throughout the protocol instead of [M] 5 micromolar (µM) blebbistatin, [M] 2 micromolar (µM) thiazovivin ([M] 10 millimolar (mM) or 5000x stock) can be used alternatively as a ROCK inhibitor. Doxycycline is light-sensitive, so keep cool (4 °C) and dark when not in use.

5 Aspirate the media from the wells and add Accutase prewarmed for 00:05:00 (the volume of Accutase to use is 1/2 the volume that you maintain the cells in). 5m

6 Put the cells with the Accutase back into the incubator for 00:12:00. 12m

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

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

9 Aspirate the supernatant and resuspend the cell pellet in 1 mL NIM + blebb.

10 Filter cells with a 35 µm or 40 µm cell strainer (for e.g., Greiner #542040).

11 Count the cells with a hemocytometer.

12 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 + doxy, LDN, CultureOne, blebb) in a 15 mL conical tube, mix well and distribute across the wells.

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.

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.

Day 1: Maintenance

13 Do nothing.

Day 2: Feed – exchange 1/3 of media

14 For 12 well plate: Add 0.5 mL **NIM +** [M] 1 µg/mL **doxy + 1xCultureOne** to each well.
For 6 well plate: Add 1 mL **NIM +** [M] 1 µg/mL **doxy + 1xCultureOne** 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

15 Do nothing.

Day 4: Feed – exchange 1/3 of media.

16 For 12 well plate: Remove 0.5ml media and replace with fresh 0.5 mL **NIM +** [M] 1 µg/mL

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

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

Day 6: Feed – exchange 1/3 of media.

17 For 12 well plate: Remove 0.5 mL media and replace with fresh 0.5 mL **BrainPhys + B27 (50x) +** [M] 1 µg/mL **doxy + BDNF (** [M] 50 ng/mL **) + GDNF (** [M] 10 ng/mL **) + NIC (** [M] 10 millimolar (mM) **).**
For 6 well plate: Remove 1 mL media and replace with fresh 1 mL **BrainPhys + B27 (50x) +** [M] 1 µg/mL **doxy + BDNF (** [M] 50 ng/mL **) + GDNF (** [M] 10 ng/mL **) + NIC (** [M] 10 millimolar (mM) **).**

Day 8: Feed – exchange 1/3 of media.

18 For 12 well plate: Remove 0.5 mL media and replace with fresh 0.5 mL **BrainPhys + B27 (50x) +** [M] 1 µg/mL **doxy + BDNF (** [M] 50 ng/mL **) + GDNF (** [M] 10 ng/mL **) + NIC (** [M] 10 millimolar (mM) **).**
For 6 well plate: Remove 1 mL media and replace with fresh 1 1 mL **BrainPhys + B27 (50x) +** [M] 1 µg/mL **doxy + BDNF (** [M] 50 ng/mL **) + GDNF (** [M] 10 ng/mL **) + NIC (** [M] 10 millimolar (mM) **).**

> Note
>
> 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).

19 For long-term experiments >1 week, continue feeding by 1/3 media exchange every other day with **BrainPhys + B27 (50x) +** [M] 1 µg/mL **doxy + BDNF (** [M] 50 ng/mL **) + GDNF (** [M] 10 ng/mL **) + NIC (** [M] 10 millimolar (mM) **).**

Media/Reagent Recipes

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

	A	B	C

	A	B	C
	Component	**Catalog #**	**Volume**
	DMEM/F12	Thermo Fisher Scientific # 11330	485 ml
	N2 supplement (100x)	Thermo Fisher Scientific # 17504044 or recipe below	5 ml
	NEAA (non-essential amino acids, 100x)	Thermo Fisher Scientific # 11140	5 ml
	GlutaMAX supplement (100x)	Thermo Fisher Scientific # 35050061	5 ml
			Total 500 ml

21 **N2 supplement (100X) recipe:**

	A	B	C	D	E	F
	Component	**100 ml**	**125 ml**	**200 ml**	**250 ml**	**500 ml**
	Transferrin	1g	1.25 g	2 g	2.5 g	5.0 g
	Insulin	50 mg	62.5 mg	100 mg	125 mg	250 mg
	Progesterone	63 µg	78.5 µg	126 µg	157 µg	315 µg
	Putrescine	161 mg	201.5 mg	322 mg	403 mg	806 mg
	Sodium Selenite	50.2 µg	62.75 µg	100.4 µg	125.5 µg	251 µg
	DMEM/F12	**to 100ml**	**to 150ml**	**to 200ml**	**to 250ml**	**to 500ml**

22 **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 (doxy) (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.

LDN-193189 hydrocholoride (BMP blocker - Noggin replacement – 10,000X stock ([M] 1 millimolar (mM)); Sigma SML0559-5MG):

g = Molecular Weight (g/mol) * Molarity (M) * Volume (ml)

0.005 g = (406.48) * (0.001 M) * (Volume)

0.005 g = 0.41 * 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 bottle overnight On ice before aliquoting. Always keep on ice and never let 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 or 0.5 mL per well of 12-well plate.

NIC (Nicotinamide) (Sigma #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 = (122.12)*(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) - 20X stock ([M] 2 mg/mL ; mol wt 30,000-70,000; Sigma P3655-500MG):

Add 500 mg PLO to 250 mL cell culture ddH2O.

Make 1 mL aliquots. Store aliquots in -80 °C.