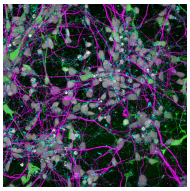
Mar 12, 2025 Version 3

hPSC Culture, iNeuron Differentiation and Culture in N2B27 vs BrainPhys for Immunofluorescence and Biochemistry Assessments of Mitophagy V.3



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DOI
https://dx.doi.org/10.17504/protocols.io.5qpvo36xxv4o/v3

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DOI: https://dx.doi.org/10.17504/protocols.io.5qpvo36xxv4o/v3

External link: https://doi.org/10.1101/2025.04.01.646648

Protocol Citation: Benjamin O'Callaghan, Helene Plun-Favreau 2025. hPSC Culture, iNeuron Differentiation and Culture in N2B27 vs BrainPhys for Immunofluorescence and Biochemistry Assessments of Mitophagy. **protocols.io** https://dx.doi.org/10.17504/protocols.io.5qpvo36xxv4o/v3Version created by **Benjamin O'Callaghan**

Manuscript citation:
Benjamin O'Callaghan, Daniela Melandri, Darija Soltic, Katharina Cosker, Marc P.M. Soutar, Helene Plun-Favreau (2025) iNeurons are sweet, maybe too sweet? Exploring the impact of media composition on PINK1-dependent mitophagy.bioRxiv doi: 10.1101/2025.04.01.646648

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

Created: February 13, 2024

Last Modified: March 12, 2025

Protocol Integer ID: 95168

Keywords: ASAPCRN, iNeuron, BrainPhys, N2B27, biochemistry assessments of mitophagy protocol, culture of wtc11 hpsc, subsequent culture in n2b27, brainphys for immunofluorescence, culture in n2b27, mitophagy protocol, brainphys medium, ineuron differentiation, inducible ngn2 system, doxycycline, wtc11 hpsc, n2b27, brainphy, ineuron, immunofluorescence

Funders Acknowledgements:
Aligning Science Across Parkinson's
Grant ID: ASAP 000478

Abstract

Protocol for the culture of WTC11 hPSCs harbouring a doxycycline-inducible Ngn2 system, the differentiation of this line into iNeurons and subsequent culture in N2B27 or BrainPhys medium.

Materials

Materials/Reagents:

- iNeuron hPSC Line (iNeuron hPSC Line established on WTC11 hPSC background with TET-ON system at the AAVS1 safe-harbour locus for overexpression of murine Ngn2 and dCas9-KRAB at the CLYBL safe harbour locus was a kind gift from the lab of Michael Ward)
- mTeSR Plus (STEMCELL Technologies, 100-0276)
- Ca^{2+}/Mg^{2+}-free Dulbecco's Phosphate Buffered Saline (DPBS) (Gibco, 14190169)
- 0.5M EDTA (Invitrogen, 15575020)
- Geltrex (Gibco, A1413302)
- TrypLE Express (Gibco, 12604021)
- Dulbecco's Modified Eagle Medium (DMEM) (Gibco, 11995-065)
- Heat-inactivated foetal bovine serum (FBS) (Gibco, A5256801)
- Y27632 ROCKi (MedChem, HY-10071)
- DMEM/F12 - HEPES (Gibco, 11330032)
- Neurobasal (Gibco, 21103049)
- DMEM, no glucose, no glutamine, no phenol red (Gibco, A1443001)
- Neurobasal-A Medium, no D-glucose, no sodium pyruvate (Gibco, A2477501)
- Glucose Solution (Gibco, A2494001)
- Pyruvate Solution (Gibco, 11360070)
- Glutamax Supplement (Gibco, 35050061)
- Non-essential amino acids (NEAA))Gibco, 35050061)
- N2 supplement (Gibco, 17502048)
- B27 Supplement (Gibco, 17504044)
- Insulin (~10mg/ml, Sigma, I9278)
- B-mercaptoethanol (50mM, Gibco, 31350010)
- Accutase (Sigma, A6964)
- BrainPhys (STEMCELL Technologies, 5790)
- BDNF (Peprotech, 450-02)
- NT-3 (Peprotech, 450-03)
- Doxycycline (Dox) (Sigma, D5207)
- Trizma hydrochloride (Tris-HCl) (Sigma, T3253)
- Sodium Hydroxide (Sigma, S5881)
- Sodium Chloride (NaCl) (Sigma, S9625)
- 10% w/v Triton-X-100 (Sigma, 93443)
- Sodium Deoxycholate (Sigma, D6750)
- Sodium Dodecyl Sulphate (SDS) (Sigma, L4509)
- PhosSTOP Phosphatase Inhibitors (Sigma, PHOSS-RO)
- cOmplete mini, EDTA-free Protease Inhibitors (Sigma, 11836170001)
- 37% Formaldehyde Solution (Sigma, F8775)
- 10x Phosphate Buffered Saline (Fisher Scientific, 10649743)
- Dimethyl sulphoxide (Sigma, 41639)
- Oligomycin (Sigma O4876)
- Antimycin A (Sigma A8674)
- PhenoPlate 96-well Microplates (Revvity, 6055308)
- Opera Phenix High-Content Confocal Microscope System
- Monarch Total RNA Miniprep Kit (New England Bioscience, T2010S)
- Nanodrop One Spectrophotometer (Thermo Fisher Scientific, ND-ONE-W)
- SuperScript IV reverse transcriptase (Invitrogen, 18090050)
- Random hexamer primers (Thermo Fisher Scientific, SO142)
- dNTPs (Thermofisher Scientific, R0191)
- RNaseOUT (Invitrogen, 10777019)
- Power SYBR Green Master Mix (Applied Biosystems, 4367659)
- UltraPure DNase/RNase-Free Distilled Water (Invitrogen, 10977035)

Buffers/Stocks:

- Geltrex is diluted 1:100 in cold serum free DMEM for coating of culture surfaces
- 0.5mM EDTA = 1:1000 dilution of 0.5M EDTA in Ca^{2+}/Mg^{2+}-free DPBS
- 5mM Y27632 ROCKi = powder dissolved in Ca2+/Mg2+-free DPBS
- 2mg/ml doxycycline = powder dissolved in Ca2+/Mg2+-free DPBS
- 10ug/ml BDNF = powder dissolved in Ca2+/Mg2+-free DPBS
- 10ug/ml NT-3 = powder dissolved in Ca2+/Mg2+-free DPBS

- 1x PBS = 1:10 dilution of 10x PBS in dH2O
- 24% formaldehyde in 1x PBS = 37% formaldehyde diluted with dH2O and 10xPBS
- 1M Tris-HCl (pH=7.4) = powder dissolved in dH2O and pH to 7.4 with sodium hydroxide
- 2M NaCl = solid dissolved in dH2O
- 10% w/v sodium deoxycholate = solid dissolved in dH2O
- 2% w/v SDS = powder dissolved in dH2O
- RIPA Lysis Buffer = 50mM Tris-HCl (pH=7.4), 150mM NaCl, 1% w/v triton-X-1OO, 0.5% sodium deoxycholate, 0.1% w/v SDS, 1x phosphatase inhibitors, 1x protease inhibitors, prepared in dH2O

Safety warnings

- Oligomycin and Antimycin are mitochondrial poisons which should be handled with appropriate PPE and disposed of through safe disposal routes
- Formaldehyde is systemic poison which should be handled with appropriate PPE and disposed of through safe disposal routes

hPSC Maintenance

1 **Maintenance of iNeuron hPSCs**
The established WTC11 iNeuron hPSC Line is maintained under standard culture conditions.
mTeSR Plus media and geltrex coated dishes.
Passaged as cell aggregates using [M] 0.5 millimolar (mM) EDTA (in Ca^{2+}/Mg^{2+}-free Dulbecco's Phosphate Buffered Saline (DPBS)) every 3-4 days to maintain subconfluent culture.
We find the WTC11-iNeuron hPSCs and derived modifications of this cell line to grow quite quickly in our hands compared to other cell lines.
Typical split ratios range from 1:6-1:12.
Maintain cells in a humidified incubator, 37 °C , 5%/95% CO_2/AIR mix

iNeuron Differentiation

4d 6h 8m

2 **d-1: Seed** 3h
The below describes a standard seeding and differentiation.
Before beginning make sure you have Geltrex coated 6-well plates (Generally aim to do Overnight but > 03:00:00 will be fine).

2.1 TrypIE split healthy and subconfluent iNeuron hPSCs into a single cell suspension.

Wash with DPBS, add TrypIE, incubate until cells are starting to detach. Aspirate TrypIE and wash cells off with DMEM 10% FBS cell culture media. Note if cells have already detached en masse in TrypIE just add DMEM 10% FBS directly to the TrypIE (don't aspirate TrypIE first).

2.2 Centrifuge 240 x g, Room temperature, 00:04:00 to pellet hPSCs 4m

2.3 Remove supernatant and resuspend pellet in mTesR Plus supplemented with [M] 10 micromolar (µM) Y-27632 ROCKi (i.e. 1:500 dilution of [M] 5 millimolar (mM) ROCKi stock).

Generally do this resuspension initially with a p1000 and 1-2ml of media in order to fully dissociate cells into single cell suspension. Then top up with mTeSR Plus +ROCKi (as a guide 1-2ml per well split)

2.4 Count viable cells and prepare a cell suspension in mTeSR Plus +ROCKi such that 0.45×10^6 cells are in 1.5 mL l (i.e. 0.3×10^6 cells per ml). You will need 1.5 mL per well being seeded

Note: As a guide, we typically find the yields of cells on d3 final plating to be approximately 2x that seeded here.

2.5 Remove geltrex from coated plates

2.6 Plate 1.5 mL of the cell suspension prepared in go to step #2.4 per well of geltrex-coated 6-well.

2.7 Place in humidified incubator, 37 °C 5%/95% CO_2/AIR mixture, Overnight

3 **d0-d2 = Induction Media**
Prepare induction media (DMEM/F12 - HEPES, 1x N2 supplement, 1x NEAAs).

I usually prepare enough Induction media without doxycycline for all 3x changes. And then aliquot and add fresh doxycycline each day.

	A	B	C
	Induction Media (IM)		
	Component:	Product #:	Amount per 50mL:
	DMEM/F12, HEPES	Gibco 11330032	49mL
	N2 Supplement, 100x	Gibco 17502048	500 ul
	Non-essential Amino Acids (NEAA), 100x	Gibco 11140050	500 ul

50ml of Induction Media without Doxycycline

3.1 Supplement Induction media from go to step #3 with [M] 2 µg/ml doxycycline (=1:1000 of [M] 2 mg/mL stock)

3.2 Assess cells seeded yesterday in go to step #2 Cells should be evenly distributed across the surface and be <50% confluent. I have found that more confluent or "clumpy" seeded cultures can give rise to incomplete neuronal differentiation and contamination of end-point cultures with non-neuronal proliferative cell types. 1d

Remove mTeSR Plus media from cells plated in go to step #2 and add 2 mL of induction media + [M] 2 µg/ml doxycycline prepared in go to step #3.1 to each well Note: I like to do this < 24:00:00 after seed in order to ensure culture doesn't get too confluent.

3.3 Place in humidified incubator, 37 °C 5%/95% CO_2/AIR mixture

3.4 24:00:00 (d1) and 48:00:00 (d2) later change with 2 mL fresh induction media + [M] 2 µg/ml doxycycline prepared as in go to step #3.1 3d

4 **d3: Split and Final Plate**

Prepare N2B27 media (1:1 mixture of DMEM/F12:Neurobasal supplemented with 0.5x N2-supplement, 0.5x B27 supplement, 0.5x NEAAs, 0.5x Glutamax, [M] 45 micromolar (µM) 2-Mercaptoethanol, [M] 2.5 µg/ml insulin) and Complete BrainPhys Media (BrainPhys supplemented with 1x B27, [M] 10 ng/ml BDNF and [M] 10 ng/ml NT-3).

	Component	Volume	Final Concentration
	DMEM/F12	24.5ml	0.5x
	Neurobasal	24.5ml	0.5x
	N2 supplement (100x)	250ul	0.5x

	Component	Volume	Final Concentration
	Insulin (10mg/ml)	12.5ul	2.5ug/ml insulin
	Non Essential Amino Acids (100x)	250ul	0.5x
	B-mercaptoethanol (50mM)	45ul	45uM
	B27 supplement	500ul	0.5x
	Glutamax	250ul	0.5x

50ml of N2B27

	Component	Volume	Final Concentration
	BrainPhys	49ml	1x
	B27 supplement	1ml	1x
	BDNF (10ug/ml)	50ul	10ng/ml
	NT-3 (10ug/ml)	50ul	10ng/ml

50ml of Complete BrainPhys

For experiments modulating glucose and pyruvate levels of N2B27 a simplified N2B27 base consisting of 1:1 mixture of DMEM, no glucose, no glutamine, no phenol red:Neurobasal-A Medium, no D-glucose, no sodium pyruvate, supplemented with 0.5x N2-supplement, 0.5x B27 supplement, 0.5x NEAAs, [M] 2 millimolar (mM) Glutamax, [M] 45 micromolar (µM) 2-Mercaptoethanol, [M] 2.5 µg/ml insulin. This base was then supplemented additionally with 21.25mM glucose and 0.36mM pyruvate (high glucose) or 2.5mM glucose and 0.5mM pyruvate (low glucose).

	Component	Volume	Final Concentration
	DMEM, no glucose, no glutamine, no phenol red	24.5ml	0.5x
	Neurobasal-A Medium, no D-glucose, no sodium pyruvate	24.5ml	0.5x
	N2 supplement (100x)	250ul	0.5x
	Insulin (10mg/ml)	12.5ul	2.5ug/ml insulin
	Non Essential Amino Acids (100x)	250ul	0.5x

	Component	Volume	Final Concentration
	B-mercaptoethanol (50mM)	45ul	45uM
	B27 supplement (50x)	500ul	0.5x
	Glutamax (100x / 200mM)	500ul	2mM
	Glucose (200g/L / 1.11M)	957ul	21.25mM
	Pyruvate (100mM)	180ul	0.36mM

50ml of High Glucose Simplified N2B27

	Component	Volume	Final Concentration
	DMEM, no glucose, no glutamine, no phenol red	24.5ml	0.5x
	Neurobasal-A Medium, no D-glucose, no sodium pyruvate	24.5ml	0.5x
	N2 supplement (100x)	250ul	0.5x
	Insulin (10mg/ml)	12.5ul	2.5ug/ml insulin
	Non Essential Amino Acids (100x)	250ul	0.5x
	B-mercaptoethanol (50mM)	45ul	45uM
	B27 supplement (50x)	500ul	0.5x
	Glutamax (100x / 200mM)	500ul	2mM
	Glucose (200g/L / 1.11M)	112.6ul	2.5mM
	Pyruvate (100mM)	250ul	0.5mM

50ml of Low Glucose Simplified N2B27

High glucose BrainPhys consisted of the Complete BrainPhys formulation outlined above with additional glucose to bring the final glucose concentration from 2.5mM to 21.25mM.

	Component	Volume	Final Concentration
	BrainPhys	49ml	1x
	B27 supplement	1ml	1x
	BDNF (10ug/ml)	50ul	10ng/ml
	NT-3 (10ug/ml)	50ul	10ng/ml
	Glucose (200g/L / 1.11M)	844ul	21.25mM

50ml of High Glucose BrainPhys

4.1 Before beginning make sure you have Geltrex coated plates (Generally aim to do Overnight but > 03:00:00 will be fine). 3h

Note: edge effects are quite commonly a problem in small well format dishes such as 96-well and to a lesser degree 24-wells. It is therefore recommended to not use the perimeter wells of such formats, and instead fill these with sterile water or PBS to maintain humidity.

4.2 Remove media from cells and wash 1x gently with DPBS

4.3 Add accutase solution to each well, sufficient to cover cell layer (0.4-0.5 mL per 6-well).
Monitor cells under microscope until cells have almost entirely detached.

4.4 Gently wash cell surface with accutase and collect in 15ml falcon.
Then wash each of the wells with DMEM F12 (just serum free base media) and collect in same falcon tube.

4.5 Top up cell suspension to 6x accutase volume with DMEM/F12 HEPES.
E.g. if you collect 4x 0.5 mL accutase = 2 mL, top up to 12 mL with DMEM F12 media.

4.6 Pellet cells at 300 x g, Room temperature, 00:04:00 and remove accutase 4m
containing supernatant. It is good to try and get rid of as much of this as possible as accutase will remain active. If you don't manage to get most of the accutase media off, add another 10 mL of F12-HEPES and pellet again to dilute further.

4.7 Resuspend cell pellets in small volume of N2B27 or BrainPhys media
I usually resuspend the pellet in 500 µL of media per 6-well split.

4.8 Count cells and prepare cell suspension at appropriate dilutions for final plating.
We routinely use 12-well formats for biochemistry assays (whole-cell lysate and RNA preps) and 96-well format for imaging based assays.

	Plate Format	Number of cells per well	Cell suspension concentration / cells/ml	Volume of cell suspension required	Culture Volume after first change
	12-well	6×10^5	6×10^5	1000	1000-1500
	24-well	3×10^5	6×10^5	500	500-750
	96-well	5×10^4	5×10^5	100	150

Recommended seeding densities and culture volumes

4.9 Remove geltrex from dishes and add desired volume of appropriate cell suspension prepared in go to step #4.8 .
It is recommended to fill any empty wells with sterile water or DPBS to minimise evaporation effects.

4.10 Place in humidified incubator, 37 °C 5%/95% CO_2/AIR mixture for 2-3hr

4.11 **d4 Onwards: Half media change**

Perform a half media change with N2B27 or BrainPhys twice a week.
I usually perform the first media change after seeding on d6.

For example:
12-well currently in 1000 µL . Remove 250 µL and add 750 µL of fresh media.
96-well currently in 100 µL . Remove 25 µL and then add 75 µL of fresh media.

I then perform the next half media change on d9.
For example:
12-well currently in 1500 µL . Remove 750 µL and add 750 µL of fresh media.
96-well currently in 100 µL . Remove 25 µL and then add 75 µL of fresh media.

And then half media change every Monday or Tuesday and Thursday or Friday thereafter.

Note it is important to be very gentle when doing this. Avoid dropping the media meniscus below full covering of plate and add media as slowly as possible to wells. Older cultures can easily detach as a sheet en masse.

Treatment and Collection

1h

5 Other differentiation time points might be interesting but we typically culture until at least d17 but have experience maintaining these cultures up until d60 without any obvious negative effects.

We always perform a half media change 24h prior to any collection time-points or assays.

Collection on d24 with a half media change on d23 represents our most typical experimental conditions.

5.1 PINK1-Dependent Mitophagy Initiation

We use a [M] 1 micromolar (µM) equimolar mixture of oligomycin and antimycin (O/A) to depolarise the mitochondrial membrane potential and trigger PINK1-dependent mitophagy. Reverse time-point experiments are performed such that all experimental conditions are collected simultaneously.

We usually prepare a [M] 11 micromolar (µM) dilution of O/A in the appropriate culture media (e.g. 11 µL of [M] 1 millimolar (mM) O/A in 1 mL media) and matching v/v dilution of DMSO (e.g. 11 µL of DMSO in 1 mL media) as a treatment control. And then add a volume = 10% of current culture volume in order to get final desired concentration.

For example:
12-well currently in 1500 µL . Add 150 µL of 11x = 1x final concentration in 1650 µL .
96-well currently in 150 µL . Add 15 µL of 11x = 1x final concentration in 165 µL .

NOTE: oligogmycin and antimycin are mitochondrial toxins which should be handled with care.

5.2 Collection for biochemistry assays

RNA:
At the appropriate treatment time-point media is removed from the culture.
Cells are then washed gently with ice-cold PBS On ice .
Ice-cold PBS is removed and cells snap-frozen in the culture plate by placing in a -80 °C freezer.

Whole-Cell Protein Lysate:
At the appropriate treatment time-point media is removed from the culture.
Cells are then washed gently with ice-cold PBS On ice .
Ice-cold PBS is removed and ice-cold RIPA cell lysis buffer placed onto the centre of the well (we usually add 50 µL - 100 µL per 12-well).
Cells are then snap-frozen in the culture plate by placing in a -80 °C freezer.

NOTE: oligomycin and antimycin are mitochondrial toxins which should be disposed of through appropriate safe disposal routes.

5.3 Fixation/Collection for Microscopy

20m

At the appropriate treatment time-point add [M] 24 % (w/v) formaldehyde (prepared in 1x PBS) to the media containing wells such that the final concentration will be [M] 4 % (w/v) formaldehyde).

For example:
96-well currently in 165 µL . Add 33 µL of [M] 24 % (w/v) formaldehyde

Incubate cells at room-temperature for 00:20:00 .
Remove formaldehyde containing media and add 200 µL of PBS for storage prior to downstream imaging/staining.

NOTE: oligomycin, antimycin and formaldehyde are toxic and should be disposed of through appropriate safe disposal routes.

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