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Imaging autophagy in hiPSC-derived midbrain dopaminergic neuronal cultures for Parkinson's disease research

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Abstract

To appreciate the positive or negative impact of autophagy during the initiation and progression of human diseases, the isolation or de novo generation of appropriate cell-types is required to support focussed in vitro assays. In human neurodegenerative diseases such as Parkinson's disease (PD), specific subsets of acutely sensitive neurons become susceptible to stress-associated operational decline and eventual cell death, emphasising the need for functional studies in those vulnerable groups of neurons. In PD, a class of dopaminergic neurons in the ventral midbrain (mDANs) is affected. To study these, human induced pluripotent stem cells (hiPSCs) have emerged as valuable tool, as these enable the production and study of mDAN biology in vitro. In this chapter, we describe a stepwise protocol for the generation of mDANs from hiPSCs using a monolayer culture system. We then outline how imaging-based autophagy assessment methodologies can be applied to these neurons, thereby providing a detailed account of the application of imaging-based autophagy assays to human hiPSC-derived mDANs.

Keywords: Autophagy; hiPSC; stem cells; dopaminergic neurons; cell culture; immunofluorescence; Parkinson's disease.

1 Introduction

Human induced pluripotent stem cell (hiPSC) technology is making major contributions to our understanding of the causes of human disease, and is a promising source of material for replacement therapies. By differentiating hiPSC into specific, disease-affected cell-types, researchers are able to investigate in vitro the molecular pathways that drive human diseases in the appropriate cell/tissue-type. This includes neurodegenerative diseases—e.g. Alzheimer's disease (AD) and Parkinson's Disease (PD)—where autophagy deregulation is a significant factor [1, 2]. For human neurodegenerative diseases, key questions centre on why particular subsets of neurons are susceptible to degeneration, either in patients genetically predisposed to the disease of in those whose condition is regarded as idiopathic. Thus, there is a need to understand disease processes in precisely those cells that are susceptible in vivo. In PD, a distinct class of ventral midbrain dopaminergic neurons (mDANs) in the substantia nigra pars compacta (SNpc) are lost. It is thought that the atypical morphology and distinctive physiology of these neurons force mDANs to operate at or close to their energy demand/supply threshold in vivo [3]. This predisposes mDANs to declining cytoplasmic quality control pathways during aging and stress. Here, we describe a protocol for the generation of mDAN cultures from hiPSCs, and methods to study autophagy in these cells using imaging. The methods can be applied equally to healthy patient-derived hiPSCs and hiPSCs from PD patients for comparative studies of underlying genetic defects, and to genome-edited hiPSC lines. We are also able to use a modified version of the protocol described here for the production of cortical neurons. Altering differentiation parameters enables researchers to push hiPSC neuralisation towards other neuronal sub-types for studies of different neurodegenerative diseases, and the reader is advised to consult focused reports for alternative neuralisation conditions (reviewed in[1]).

2 Culturing human induced pluripotent stem cells (hiPSCs)

Successful neural differentiation depends to a great extent on the quality of the human induced pluripotent stem cells (hiPSCs) that are used. Thus, their growth and expansion must be carried out meticulously, and very carefully monitored before neuralisation can be attempted (See Fig. 1). In our lab, hiPSCs are maintained in fully defined xeno-free and feeder-free complete Essential 8 (E8) Medium [4].

Notes:

- We describe using E8 medium for feeder-free hiPSC culturing. Other feeder-free systems are available, although we have not carried out direct comparisons.
- This is an indicative method for culturing hiPSCs. Minor adjustments maybe required depending on the cell-line and the age of the cells (early or late passage).

- All procedures must be carried out under sterile conditions.
- Cells or culture surfaces must not be allowed to dry out at any point.
- It is highly recommended to review the suppliers' webpages for product and method updates.

2.1 Materials

Reagents	**Provider**	**Catalogue Number**
Essential 8 medium/E8 supplement	Thermo Fisher Scientific	A1517001
Vitronectin	Thermo Fisher Scientific	A1413301
EDTA*	Thermo Fisher Scientific	15575-020
DPBS*	Thermo Fisher Scientific	14190-144
Y-27632 dihydrochloride	Tocris	1254
RevitaCell	Thermo Fisher Scientific	A26445
CryoStor CS10	Stem Cell Technologies	07930

Table 2.1: List of reagents used for the Essential 8 culturing system.

**EDTA: ethylenediaminetetraacetic acid; DPBS: Dulbecco's phosphate-buffered saline.*

2.2 Preparation of reagents

2.2.1 Complete Essential 8 medium

Complete E8 medium consists of 500ml E8 basal medium (store at 2-8°C) and 10ml E8 supplement (50X; store at -5°C to -30°C; thaw at room temperature, NOT at 37°C). The manufacturer suggests adding 10ml E8 supplement in 490ml of E8 basal medium to make 500ml of complete E8 medium; however, the complete E8 can be stored for only up to two weeks at 2-8°C. For longer storage time, we recommend decanting the E8 basal medium into 49ml aliquots (store at 2-8°C), and the E8 supplement into 1ml aliquots (refreeze at -5°C to -30°C). Before use, mix one aliquot of each to make 50ml of complete E8. We store the unmixed reagents for up to 3 months without any noticeable loss of activity.

2.2.2 Vitronectin

Vitronectin is supplied at 500μg/ml. Upon receipt, thaw at room temperature, then decant into 20μl aliquots, refreeze and store at -80°C. Prior to use, thaw and dilute vitronectin 1:100 in DPBS (e.g. 20μl vitronectin in 1980μl DPBS) and use immediately. Do not refreeze.

2.2.3 EDTA

Dilute 50μl of 0.5M EDTA to 50ml of DPBS (final concentration of 0.5mM), filter-sterilise and store in room temperature for up to 6 months.

2.3 Plating hiPSC

2.3.1 Vitronectin plate coating

1) Thaw and dilute vitronectin in DPBS as described in 2.2.2.
2) Apply diluted vitronectin to cell culturing dishes at a final concentration of ~0.5µg/cm^2 (e.g. 500µl/well of a 12-well plate; 1ml/well of a 6-well plate; 2ml in a 60mm diameter dish).
3) Coat the dishes for at least 1h at room temperature. If not to be used immediately, parafilm-shield the vitronectin containing dishes to prevent evaporation and keep them for up to 7 days at 2-8°C. Before use, ensure that the plates have not dried out, and allow them to reach room temperature (about 20 min).

2.3.2 Thawing hiPSCs

1) While waiting for the dishes to be coated, warm an appropriate volume of complete E8 medium at room temperature for approximately 30 min.
2) After coating is completed, remove a cryovial of hiPSCs from liquid nitrogen and thaw in a 37°C water bath for around 1 min.
3) Before being completely thawed, add 1ml of warm complete E8 medium to the cells.
4) Gently transfer the cells into a 15ml falcon tube containing 3ml of complete E8 medium, and centrifuge (100*g* for 3 min at room temperature).
5) Aspirate the medium, and gently resuspend the pelleted cells in 1ml of fresh complete E8 medium. It is important to not over-triturate the cells, as they must be plated in the form of small aggregates/clusters (and not as single cells) in order to thrive.
6) Aspirate the vitronectin solution and plate the cell suspension on to the coated dishes at a density of 4-5x10^4 cells/cm^2 in complete E8 medium supplemented with 1X RevitaCell (1:100) for the first 24 hours to improve cell survival (final complete E8 medium volumes: 1ml/well for 12-well plates; 2ml/well for 6-well plates; 4ml/60mm dish).
7) Gently agitate the plates to ensure equal cell distribution and incubate at 37°C, 5% CO_2.
8) Change media daily. No additional RevitaCell is required.

***Notes**:*

- Vitronectin does not need to be washed off the tissue culture surface prior to plating cells.
- The manufacturer suggests using RevitaCell (Step 6); however, 10µM Y-27632 (ROCK inhibitor) can be used as an alternative.
- Do not remove any cells undergoing spontaneous differentiation during cell expansion; these will be eliminated though consecutive passaging.

2.4 Passaging hiPSCs

hiPSCs cells need to be passaged when they reach approximately 80% density, or 4 to 5 days after initial plating (see Fig. 1D):

1) Coat culture dishes with vitronectin for 1 hour (see 2.3.1), and warm complete E8 medium.
2) When coating is complete, aspirate spent media from hiPSC cultures and wash once with 0.5mM EDTA or PBS (500µl/well of a 12-well plate; 1ml/well of a 6-well plate; 2ml/60mm diameter dish) to remove growth medium residues.
3) Replace with fresh 0.5mM EDTA (500µl/well of a 12-well plate; 1ml/well of a 6-well plate; 2ml/60mm diameter dish) and incubate for 5-8 min at room temperature or 3-5 min at 37°C.
4) When colonies start loosening and rounding up, the cells are ready to be harvested (Fig. 1E). The next 2 steps must be carried out as quickly as possible.
5) Aspirate EDTA and wash once with pre-warmed complete E8 medium.
6) Immediately aspirate the media as it neutralises the EDTA very quickly, and gently harvest the colonies by liquid flow using either a 1ml or a 5ml serological pipette.
7) Collect the dislodged cells into 15ml falcon tubes.
8) Triturate the cell suspension up to 3 times to break the colonies into smaller clusters (Fig. 1F). Do not over triturate as very small clusters and single cells do not survive passaging.
9) Aspirate vitronectin from the newly coated dishes.
10) Passage hiPSC cell suspension at a dilution of 1:4 (approximately 4-5x10^4 cells/cm^2) into complete E8 medium to a final volume of 1ml/well of 12-well plates, 2ml/well of 6-well plates, or 4ml/60mm diameter dish.
11) Gently shake the plates for equal cell distribution of cells and incubate at 37°C, 5% CO_2. If the next day the cell density is too high or too low, adjust future passaging ratios accordingly.
12) Feed daily until the cells are ready to be passaged again.

Notes:

- Cells must be split after a maximum 5 days, even if they have not reached the desired density. On the contrary, if over confluent, cells should be split earlier; however, we do not recommend passaging hiPSCs within 48 hours of plating.
- The manufacturer does not suggest washing after the EDTA treatment (Step 5); however, we have noticed that a quick wash with complete E8 medium increases cell survival.
- During harvesting (Steps 6 and 7) do not over triturate, do not scrape, and do not intensively force any cells that have not detached under gentle liquid flow.
- RevitaCell or Y-27632 are not required in established lines; however, if the cells do not recover after 2-3 consecutive passages, add 1X RevitaCell (1:100) or 10µM Y-27632 on the day of passage only.

Troubleshooting:

If the cells do not detach readily from the dish, repeat Steps 2-7, but incubate with EDTA for longer. On the contrary, if the cells detach during EDTA incubation, do not aspirate but neutralise the EDTA with 3X volume of complete E8 medium. Gently collect all the cells from the dish with a 5ml serological pipette into a 15ml falcon tube, centrifuge (100*g* for 3min at room temperature), resuspend the pellet in fresh complete E8 medium, and progress to Step 9.

2.5 hiPSC cryopreservation

1) Prepare a cryo 1°C freezing container (e.g. Mr Frosty^{TM} from ThermoFisher Scientific). "Mr Frosty" is an isopropanol filled cryovial container that upon freezing has the property of gradually decreasing temperature at a rate of 1°C/min.
2) Harvest the hiPSC as described in 2.1.3 (Steps 1-7) and collect them in 15ml falcon tubes.
3) Count the cells using a haemocytometer.
4) Centrifuge at 100*g* for 3 min at room temperature.
5) Aspirate spent media.
6) Gently resuspend cell pellet in CryoStor CS10 (~1x10^6 cells per ml).
7) Mix very gently with the tip of 1ml pipette (triturate up to 3 times) and distribute into cryovials.
8) Immediately place the cryovials into "Mr Frosty" (room temperature) and freeze at -80°C overnight.
9) Transfer the frozen cryovials into liquid nitrogen for long-term storage.

Notes:

- hiPSCs are very sensitive to temperature fluctuations. Use dry ice to transfer the cryovials between different locations.
- Cells can also be frozen in complete E8 medium supplemented with 10% dimethyl sulphoxide (DMSO); however, we have noticed decreased cell survival after thawing by this approach.
- Refill "Mr Frosty" with isopropanol every 3 uses.
- We routinely keep frozen hiPSCs at -80°C for up to 2 weeks or in liquid nitrogen for >6 months without affecting cell survival after thawing

3 hiPSC Differentiation into midbrain dopaminergic neurons (mDANs)

In order to produce hiPSC-derived neurons that are suitable for the investigation of intracellular degradation pathway function and dysfunction in PD-related research, we have optimised strategies from the literature [5-7] to develop a differentiation protocol that generates sufficient numbers of uniformly-dispersed mDANs to perform whole-cell (soma and neurites) autophagy imaging assays. At a glance, hiPSCs acquire neural identity by blocking transforming growth factor β (TGFβ) pathways [8] via

inhibition of SMAD signalling [9], while the midbrain dopaminergic fate is induced via activation of sonic hedgehog (SHH) and WNT signalling cascades that determine dorsal/ventral and anterior/posterior patterning of neural progenitors [10].

3.1 Materials

Reagent	Provider, Cat. Num.	Preparation and Storage
Accutase	Thermo F.S., A11105-01	Store at 2-8°C.
Ascorbic acid	Sigma, A5960	0.2M stock solution in dH_2O. Store 50µl aliquots at -20°C.
β-mercaptoethanol	Thermo F.S., 31350-010	50mM. Store at 2-8°C.
B27	Thermo F.S., 17504-044	Store 500µl aliquots at -20°C.
Human recombinant BDNF	R&D Systems, 248-BD or PeproTech 450-02	Make 20µg/ml stock solution in PBS, supplemented with 0.1% BSA. Store 50µl aliquots at -20°C.
Recombinant human GDNF	R&D Systems, 212-GD or PeproTech 450-10	Make 20µg/ml stock solution in PBS, supplemented with 0.1% BSA. Store 50µl aliquots at -20°C
cAMP dibutyryl (db-cAMP)	Sigma, D0627	500mM stock solution in dH_2O. Store 50µl aliquots at -20°C.
CHIR99021	Axon Medchem, 1386	600mM stock solution in DMSO. Store 50µl aliquots at -20°C.
CryoStor CS10	Stem Cell Tech., 07930	Store at 2-8°C.
DAPT	Tocris, 2634	10mM stock solution in DMSO. Store 50µl aliquots at -20°C.
DMEM/F-12 + Glutamax	Thermo F.S., 31331-028	Store at 2-8°C.
Glutamax	Thermo F.S., 35050-038	Store at 2-8°C.
Human insulin	Sigma, I9278	10mg/ml. Store at 2-8°C.
Laminin	Sigma, L2020	Store 50µl aliquots at -20°C.
LDN193189 HCl	Sigma, SML0559	1mM stock solution in dH_2O. Store 5-10µl aliquots at -80°C.
N2	Thermo F.S., 17502-048	Store 250µl aliquots in -20°C.
NEAA	Thermo F.S., 11140-035	Store at 2-8°C.
Neurobasal	Thermo F.S., 21103-049	Store at 2-8°C.

Pen/Strep	Sigma, P4333	Store at 2-8°C.
Polyornithine	Sigma, P4957	Store at 2-8°C.
SB431542	Tocris, 1614	10mM stock solution in of DMSO Store 50µl aliquots at -80°C.
Recombinant human SHH C24II	R&D Systems, 1845SH025	100µg/ml stock solution in PBS, supplemented with 0.1% BSA. Store 20µl aliquots at -20°C.
Y-27632	Tocris, 1254 or Axon, 1683	10mM stock solution in dH_2O (or DMSO). Store 50µl aliquots at -20°.

Table 3.1: List of reagents for neural differentiation

BDNF: brain derived neurotrophic factor; BSA: bovine serum albumin; cAMP: cyclic adenosine monophosphate; DAPT: N- [N- (3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester; DMEM: Dulbecco's modified Eagle's medium; DMSO: dimethyl sulphoxide; GDNF: glial derived neurotrophic factor; LDN193189: 4-[6-[4-(1-piperazinyl)phenyl]pyrazolo[1,5-a]pyrimidin-3-yl]-quinoline hydrochloride; NEAA: non-essential amino acids; Pen/Strep: Penicillin/Streptomycin; SB431542: 4-(5-benzol[1,3]dioxol-5-yl-4-pyrldin-2-yl-1H-imidazol-2-yl)-benzamide hydrate; SHH: sonic hedgehog.

3.2 Basic neuralisation medium (N2B27) recipe

Reagents	**Concentration**	**Example for 50ml N2B27**
DMEM/F-12 + Glutamax	48%	24ml
Neurobasal	48%	24ml
Glutamax	0.5%	250µl
NEAA	1%	500µl
PenStrep	1%	500µl
N2	0.5%	250µl
B27	1%	500µl
Insulin	5µg/ml	25µl
β-mercaptoethanol	75µM	75µl

Table 3.2: N2B27 medium recipe

DMEM: Dulbecco's modified Eagle's medium; NEAA: non-essential amino acids; Pen/Strep: Penicillin/Streptomycin.

Notes:

- From this point onwards, basic neuralisation medium will be referred to as N2B27.

- For providers and catalogue numbers see Table 3.1

3.3 Plate and coverslip preparation

3.3.1 Plate coating

1) Add polyornithine (500µl/well of a 12-well plate, 1ml/well of a 6-well plate or 2ml/6cm diameter dish).
2) Incubate for 2 hours at 37°C
3) Remove polyornithine. Do not wash.
4) Add laminin diluted 1:100 in DPBS (10µg/ml) (500µl/well of a 12-well plate, 1ml/well of a 6-well plate or 2ml/60mm diameter dish).
5) Incubate for 2 hours at 37°C.

Notes:

- If using tissue culture coated plates, proceed directly to Step 4 and omit the polyornithine coating step.
- If not using immediately, parafilm-shield the polyornithine or laminin containing plates to prevent evaporation. These can be kept for up to 7 days at 2-8°C. Before use ensure that the plates have not dried out and incubate for 15-20 min at 37°C.
- We reuse polyornithine and laminin solutions twice (i.e. three uses in total), without having noticed any loss of their activity.

3.3.2 Coverslip coating

1) Transfer dry-autoclaved borosilicate glass coverslips (No.1 thickness; 13mm diameter) in 4- or 24-well plates using sterile tweezers.
2) Add water or DPBS to the empty spaces between the wells to ensure humidity is maintained.
3) Add a 70µl drop of polyornithine in the centre of each coverslip.
4) Incubate for 2 hours at 37°C.
5) Remove polyornithine and without washing, add a 70µl drop of DPBS diluted laminin (dilution: 1:100, 10µg/ml) to each coverslip.
6) Incubate for 2 hours at 37°C.

Notes:

- If the droplet breaks, flood the well with another 150µl of polyornithine and/or laminin (Step dependent). Press down the coverslip with a 200µl pipette tip.
- Coverslips can be pre-prepared and stored at 2-8°C for up to 3 days, after carefully parafilm-shielding the plates without disturbing the droplets. Before use, ensure that the droplets have not dried out and incubate at 37°C for 15-20 min.

- Use the same protocol when treating live-cell imaging dishes (Section 4.2).

3.4 mDAN differentiation protocol (Fig. 2)

The efficiency of neural differentiation depends on the quality of the hiPSC culture. Consistency in hiPSC maintenance and expansion leads to reproducible neural differentiations. We recommend comparing/contrasting experimental conditions and conducting biological repeats with hiPSCs of similar passage number, otherwise epigenetic changes in hiPSCs must also be considered [11]. Newly reprogrammed hiPSCs must be checked for karyotypic abnormalities prior to using them for neural differentiation or functional studies.

General considerations:

- Do not start neuralisations with freshly-thawed hiPSCs. Instead, allow them to recover and settle for at least two consecutive passages, and ensure that the hiPSCs look similar to those shown in (Fig. 1).
- The concentrations of SHH and CHIR are crucial factors in obtaining cultures with high proportions of mDANs. Concentration ranges of 200-500ng/ml for SHH and 0.6-1.0μM CHIR are typically stated in publications [6, 7, 10].
- The protocols outlined below are designed to generate high numbers of mDANs. It is possible to generate cortical neurons following the same protocols described here, but in the absence of SHH and CHIR [12]. This can provide a useful comparison when examining mDAN biology.

3.4.1 hiPSC mDAN neuralisation procedure

1) Passage hiPSCs as described in Section 2.4, but at Step 8 triturate the cell suspension 5 times to produce smaller clusters of cells. This action produces higher numbers of smaller colonies, which we find results in higher proportions of mDANs in the final culture.
2) Depending on the needs of the particular hiPSC line, after up to ~4 days in complete E8 medium, cells are transferred to N2B27 media. Remove spent E8 medium and wash twice with pre-warmed N2B27 (500µl/well of a 12-well plate, 1ml/well of a 6-well plate or 2ml/60mm diameter dish).
3) Aspirate and replace with N2B27 supplemented with 100nM LDN, 10µM SB, 200-500ng/ml SHH, and 0.6-1.0µM CHIR (1ml/well of 12-well plates, 2ml/well of 6-well plates or 4ml/6cm diameter dish) and incubate at 37°C, 5% CO_2. This is neuralisation day 0 (DN = D0). Change media daily.
4) At D3 the cells will be passaged for the first time after the neuralisation was initiated. At least 4h before splitting the cells, prepare the plates as described in 3.3.1 (plating on to coverslips will be required after D16 for imaging studies).

5) Following laminin coating, remove spent media from the cells to be passaged and wash once with pre-warmed accutase or DBPS (500µl/well of a 12-well plate, 1ml/well of a 6-well plate or 2ml/60mm diameter dish).
6) Aspirate and replace with fresh accutase (500µl/well of a 12-well plate, 1ml/well of a 6-well plate or 2ml/60mm diameter dish) and incubate for 5min at 37°C, 5% CO_2.
7) Gently force the cells away from the plate surface by agitating the accutase once only by drawing up and down using a 1ml pipette tip. If the cells are easily detached, continue to the next step. If not, incubate for another 5 min at 37°C, 5% CO_2 and repeat the process.
8) Add an equal volume of N2B27 and gently agitate the cells 1-3 times using a 1ml pipette tip.
9) Collect the cell suspension into a 15ml falcon tube.
10) Add fresh N2B27 in the dish and repeat Steps 8 and 9 to collect any remaining cells.
11) Repeat Step 10 until all of the cells have been harvested.
12) Add additional N2B27 to the falcon tube until reaching a N2B27:accutase volume ratio of 3:1.
13) Centrifuge at 100*g*/3 min at room temperature to wash out the accutase.
14) Aspirate spent media and break up the pellet in 1ml fresh N2B27 by gently triturating for up to 5 times using a 1ml pipette tip.
15) Remove laminin from the coated plates, and plate the cells at a density of approximately $4x10^4/cm^2$ (passaging ratio of 1:2 to 1:5) in N2B27 supplemented with 100nM LDN, 10µM SB, 200-500ng/ml SHH, and 0.6-1.0µM CHIR and 10µM Y-27632 (1ml/well for 12-well plates, 2ml/well for 6-well plates or 4ml/60mm diameter dish). Incubate at 37°C, 5% CO_2. Change media daily without further addition of Y-27632.
16) At D7 the cells will need to be passaged for a second time. Repeat Steps 4-15
17) At D11, the neural inducing (SB and LDN) and patterning (SHH and CHIR) factors need to be removed from the neuralisation media. Without washing, aspirate media and add N2B27 supplemented with 20ng/ml BDNF, 20ng/ml GDNF and 0.2mM ascorbic acid (1.5ml/well of 12-well plates, 3ml/well of 6-well plates or 6ml/6cm diameter dish). Incubate at 37°C, 5% CO_2. If the next day the cells occupy the entire surface of the dish, move to the next step. If not, allow them to form a complete monolayer before proceeding to the next step, changing the media daily.
18) At D12 (or when a full monolayer is formed), passage the cells following Steps 4-15 in the appropriate amount of N2B27 supplemented with 20ng/ml BDNF, 20ng/ml GDNF, 0.2mM ascorbic acid and 10µM Y-27632, and incubate at 37°C, 5% CO_2 on laminin coated plates and incubate at 37°C, 5% CO_2. Change the media every second day or sooner if needed.
19) At D16 (or when a full monolayer is formed again) passage the cells for the fourth time (see Step 18). From this passage onwards, cells can be plated on coverslips if required (see Section 3.4.3).

20) Keep expanding and passaging the cells as described in Step 18 until D50, but not more than once per week. Change media when needed (judged by colour change), at least once every 3 days.

***Notes*:**

- Do not let the cells dry at any stage—during feeding, leave a small amount of spent media covering the cells and add the fresh media on top.
- If the day following plating cells are sparse or patchy, add 1:1000 volume of laminin.
- Cell death after passaging is expected; however, if extensive, replace with media supplemented with 10µM Y-27632 and 1:1000 laminin the day after passaging.
- The suggested cell densities are indicative. Until familiarising with the process and the particular properties of the cell-line, we recommend optimising by plating the cells at several different densities.

3.4.2 Maintaining a neural culture beyond D50

1) To split the cells after D50, repeat Steps 5-6 of section 3.4.1, and proceed to the next step.
2) Gently, remove accutase and wash once with fresh N2B27. Do not use an aspirator.
3) Remove the wash media and harvest the cells by dislodging them using a 1ml pipette tip and 1-2ml of fresh N2B27 supplemented with 20ng/ml BDNF, 20ng/ml GDNF, 0.2mM ascorbic acid, 1: 1,000 laminin, and 10µM Y-27632. Collect them in a 15ml falcon tube, but do not pellet by centrifugation.
4) Triturate the cells gently up to 7 times with a 1ml pipette tip and plate them in laminin-coated plates (see 3.3.1 section) at a density of approximately $10^5/cm^2$.
5) Passage cells after they have established a full monolayer, but not more frequently than every 10 days (and every 15 days after neurons have reached D100). Change media when needed or at least every 4 days with N2B27 supplemented with 20ng/ml BDNF, 20ng/ml GDNF and 0.2mM ascorbic acid, adjusting the medium volume accordingly. Add 1:1000 laminin every second feeding. Adhering to this methodology, we have kept the neurons in culture for more than a year.

3.4.3 Passaging on coverslips: terminal differentiation

For imaging-based investigations, we recommend plating neuralised cells on coverslips from D16 onwards. However, cells can be plated on coverslips earlier should analysis of early differentiation parameters be required.

1) Coat coverslips with polyornithine and laminin as described in 3.3.2.

2) Harvest the neural progenitors as described in Section 3.4.1, Step 19. Collect in a 15ml falcon tube, centrifuge at 100*g*/3min at room temperature and resuspend the pellet in a maximum volume of 2ml of fresh N2B27 supplemented with 20ng/ml BDNF, 20ng/ml GDNF, 0.2mM ascorbic acid and 10µM Y-27632 to initiate terminal differentiation.
3) Gently, triturate up to 5 times with a 1ml pipette tip.
4) Remove laminin droplets from the pre-coated coverslips and replace with 100µl droplet of N2B27 supplemented with 20ng/ml BDNF, 20ng/ml GDNF, 0.2mM ascorbic acid, 500µM db-cAMP, 10µM DAPT and 10µM Y-27632.
5) Add 5-10,000 cells per droplet (approximately 5,000 cells/cm^2). This amount of cells is usually contained within 10-50µl of cell suspension, depending on how much media was used to resuspend the pellet in Step 2.
6) With care so as to not disturbed the droplets, incubate at 37°C, 5% CO_2 for 1 hour to allow cells to settle and attach.
7) Flood the wells by topping them up with 350µl of N2B27 supplemented with 20ng/ml BDNF, 20ng/ml GDNF, 0.2mM ascorbic acid, 500µM db-cAMP, 10µM DAPT and 10µM Y-27632.
8) Before incubating at 37°C, 5% $CO_{2,}$ ensure that the coverslips are not floating after flooding by gently pushing them down with a pipette tip.
9) Feed the cells at three day intervals by removing 250µl spent medium and replacing with 350µl fresh N2B27 supplemented with 20ng/ml BDNF, 20ng/ml GDNF, 0.2mM ascorbic acid, 500µM db-cAMP and 10µM DAPT. Thereafter, continue the 3-day feeding regime, adding 1:1000 laminin every second feeding (no additional Y-27632 is required.)
10) After 30 days on coverslips, completely remove DAPT from the feeding media and feed once every 5 days. In this way, we have kept neurons on the same coverslips for more than 3 months.

Notes:

- Do not use aspirators to remove spent media.
- The suggested cell densities are indicative. To familiarise with the process and the cell-line behaviour, we recommend optimising by plating the cells in several different densities.
- If any droplet breaks while plating, flood the well with another 350µl media and add the double amount of cells.
- To ensure equal cell distribution after adding the cells in the droplet, very gently triturate both together once on the coverslip with a 200µl pipette tip (one up/down).
- Cells can stay in droplets overnight; however, if adopting this approach ensure that the empty spaces between the wells are field with sterile water or DPBS to increase the humidity inside the plate. Flood the wells first thing in the next morning (see Step 7 above).

3.5 Cryopreservation and thawing of neural progenitors

We cryopreserve neural progenitors from differentiation D6 until ~D60. Beyond D60, progenitors can be frozen, but cell survival (after thawing) is diminished. We suggest (after D60) freezing 3x more progenitors than is recommended at earlier time-points (outlined below).

3.5.1 Cryopreservation

1) At differentiation D16 prepare a “Mr Frosty” and follow the process as described in Section 2.5.
2) After centrifuging and removing the spent media and accutase, resuspend the pellet in CryoStor CS10 (~$1x10^6$ cells per ml) triturating up to 3 times.
3) Distribute in cryovials and follow the same process as described in Section 2.5.

3.5.2 Thawing

1) Prepare laminin coated plated as described in 3.3.1
2) Thaw cell by placing cryovials in a 37°C water bath for up to 1 min.
3) Before they are completely thawed, add 1ml of warm N2B27 per vial.
4) When completely thawed, add another 2ml of N2B27 to dilute the CryoStor.
5) Centrifuge (100*g*/3min at room temperature) to wash out the freezing medium.
6) Aspirate the medium and gently resuspend the cells in 1ml of fresh N2B27, triturating up to 5 times.
7) Remove laminin from the pre-coated plates and add the appropriate amount of N2B27 (1ml/well for 12-well plates, 2ml/well for 6-well plates or 4ml/60mm diameter dishes) supplemented with 20ng/ml BDNF, 20ng/ml GDNF, 0.2mM ascorbic acid and 10µM Y-27632. After this initial step, inclusion of Y-27632 is not recommended.
8) Add cell suspension to plates and gently agitate for equal cell distribution. Incubate at 37°C, in 5% CO_2. Replace with fresh N2B27 supplemented with 20ng/ml BDNF, 20ng/ml GDNF and 0.2mM ascorbic acid the following day if necessary.
9) Feed the cells when needed but at least once every three days with N2B27 supplemented with 20ng/ml BDNF, 20ng/ml GDNF and 0.2mM ascorbic acid.

***Notes*:**

- Do not recover the cells directly on to coverslips. Allow them to settle on coated cell culture dishes and readapt for one passage

4 Imaging-based methods to monitor autophagy in mDAN cultures

In the following sections, we outline approaches to image (macro)autophagy in hiPSC-derived mDANs. In theory, imaging approaches that are used to study autophagy in common laboratory cell-line models

can be transferred to these cells, but caution is needed when working with such fragile cells. In addition, certain typical cell fixation and staining techniques are less successful when using hiPSC-derived mDANs, so pilot studies are always necessary to find the best conditions.

Notes:

- Researchers wishing to stimulate autophagy in hiPSC-derived neuronal cultures need to test whether cells are responsive to the inducer. In our hands, amino acid starvation is less effective than pharmacological mTOR inhibition (using e.g. AZD8055).
- In all experiments, it is essential that autophagic flux is taken into account, since an increase in autophagosomes might indicate upregulated autophagy and/or reduced lysosomal clearance.

4.1 Fixed cell imaging for autophagy analysis in mDAN-enriched hiPSC neuronal cultures

In autophagy research, image-based quantitation of autophagosomes and autophagosome assembly sites provides researchers with a rapid and direct means for measuring autophagic activity [13]. Careful flux analysis is particularly important during static, fixed cell autophagy analysis, since the location of autophagosome assembly with respect to the positioning of the lysosomal pool might means that impaired autophagosome retrograde trafficking will alter flux kinetics (for discussion, see [1]).

Notes:

- For fixed cell neuronal imaging, it is important to confirm that cells analysed are of the correct neuronal classification (e.g. for mDANs, this is best done by counterstaining with anti-tyrosine hydroxylase (TH) antibodies and for midbrain markers e.g. FOXA2 (see Fig. 3A)).
- As with any immunofluorescence methodology, the fixation method must be carefully considered to allow accurate preservation of neuronal structure, and antigenicity.
- Preservation of neuronal fine structure is difficult with methanol, so care must be taken to ensure that coverslips do not dry out when transferring from aqueous to solvent and vice versa. Equally, coverslips should not be too wet so as to avoid mixing disruption when transferring to methanol.
- To analyse autophagy, we use anti-WIPI2 antibodies to detect assembly sites (see [1]. In our hands, anti-LC3 antibodies are less than satisfactory, because it is often hard to distinguish cell debris from “true” autophagosomes in these cultures. This is particularly an issue when analysing autophagosomes within neurites—visualisation with anti-LC3 antibodies is more straightforward when observations are restricted to the soma.
- For a rapid and reliable method to visualise autophagosomes and lysosomes, we co-stain with CytoID (Fig. 3C) and LysoTracker (see [1]).
- We recommend that antibody and dye staining is carried out in the plate in order to preserve neuronal structure.

- We store Mowiol in 1ml aliquots at -20°C. As needed, thaw and add 25mg DABCO antifade and store for up to 6 weeks at 4°C.
- We recommend incubating with antibodies overnight at 4°C, whilst ensuring that the samples do not dry out.
- For a general summary of immunofluorescence techniques as applied to standard cell-line models, see [14].

4.1.1 Materials

Reagent	Provider, Cat. Num.	Preperation and Storage	Working concentration & incubation length
AZD8055	Stratech, S1555	1mM stock solution in DMSO. Store at -20°C	1µM for 2-4h
Rapamycin	Calbiochem, 53123-88-9	1mM stock solution in DMSO. Store at -20°C	1µM for 2-4h
Bafilomycin	Enzo, CM110-0100	20µM stock solution in DMSO. Store at -20°C	20nM for at least 1h
Leupeptin	Sigma, L2884	20mM stock solution in DMSO. Store at -20°C	20µM for 6h or 10µM overnight.

Table 4.1: Reagents for autophagy induction and flux assessment hiPSC neuronal cultures.

Reagent	Provider, Cat. Num.	Preparation and Storage	Working Concentration
Paraformaldehyde (PFA)	Thermo F.S., 28908	1 ampule content diluted in 30ml PBS. Store at 2-8°C.	4%
Bovine Serum Albumin (BSA)	Sigma, A9418	BSA solutions are prepared in PBS. Store at 2-8°C.	5% or 2.5%
Triton X-100	Sigma, X100	Triton X100 is diluted in PBS. Store at room temperature	0.3% or 0.15%
Saponin	Sigma, S4521	Prepare 10% stock solution in PBS. Store at 2-8°C for up to 4 weeks.	0.1% for blocking and antibody solutions
Donkey serum	Sigma, D9663	Store aliquots at -20°C.	10%
Mowiol 40-88	Sigma, 324590	Store in 1ml aliquots at -20°C. Defrost, add 25mg DABCO and store at 4°C.	N/A

DABCO	Sigma, D2522	Store at 2-8°C.	25mg/ml
Live-cell imaging dishes	MatTek, P35G-0.170-14-C	N/A	N/A

Table 4.2: Reagents for fixation, permeabilisation, blocking and mounting of hiPSC neuronal cultures.

Reagent	**Provider, Cat. Num.**	**Fixation**	**Working dilution & incubation length**
CytoID	Enzo, ENZ-51031	PFA	2µl/ml for 30 min in live cells
LysoTracker Red	Thermo F.S., L7528	PFA	0.5µM for 20 min in live cells
LysoTracker Deep Red	Thermo F.S., L12492	PFA	0.5µM for 20 min in live cells
MitoTracker Red	Thermo F.S., M7512	PFA	0.5µM for 20 min in live cells
MitoTracker Green	Thermo F.S., M7514	PFA	0.5µM for 20 min in live cells
MitoSOX	Thermo F.S., M36008	PFA	5µM for 20 min in live cells
Hoeschst 33342	Thermo F.S., 62249	PFA/methanol	Iµg/ml for 5 min in live cells
DAPI	Sigma D9542	PFA/methanol	0.1µg/ml for 45 min with fixed cells
Anti-TH (rabbit)	Millipore, AB152	PFA/methanol	1:300. Incubate for 2 hours-to-overnight
Anti-TH (mouse)	Santa Cruz, sc-25269	PFA/methanol	1:100. Incubate for 2 hours-to-overnight
Anti-βIII tubulin (TUJ1)	Bio-legend, 801201	PFA/methanol	1:1000. Incubate for 2 hours-to-overnight
Anti-FOXA2	Santa Cruz, sc-101060	PFA/methanol	1:100. Incubate for 2 hours-to-overnight
Anti-LMX1A	Millipore, AB10533	PFA/methanol	1:1000. Incubate for 2 hours-to-overnight
Anti-LMX1B	Proteintech, 18278	PFA fixation	1:250. Incubate for 2 hours-to-overnight
Anti-TBR1	Abcam, AB31940	PFA fixation	1:500. Incubate for 2 hours-to-overnight
Anti-WIPI2 (mouse monoclonal)	BioRad, MCA5780GA	Methanol	1:400 Incubate for 1 hour
Anti-LC3B (rabbit polycolonal)	Sigma, L7543	Methanol	1:400. Incubate for 1 hour

Anti-P62 (mouse monoclonal)	Abnova, H00008878	PFA/Methanol	1:400. Incubate for 1 hour
Anti-LAMP1	DSHB, H4A3-5	PFA and saponin	1:400. Incubate for 2 hours-to-overnight
Donkey Anti-Rabbit Alexa Fluor 488	Thermo F.S., A21206		1:400. Incubate for 1 hour
Donkey Anti-Rabbit Alexa Fluor 568	Thermo F.S., A10042		1:400. Incubate for 1 hour
Donkey Anti-Mouse Alexa Fluor 488	Thermo F.S., A21202		1:400. Incubate for 1 hour
Donkey Anti-Mouse Alexa Fluor 488	Thermo F.S., A10037		1:400. Incubate for 1 hour
Donkey Anti-Rabbit Alexa Fluor 647	Thermo F.S., A31573		1:400. Incubate for 1 hour
Donkey Anti-Mouse Alexa Fluor 647	Thermo F.S., A31571		1:400. Incubate for 1 hour

Table 4.3: Antibodies and associated reagents for fluorescence analysis of hiPSC neuronal cultures

4.1.1 Methanol fixation for immunofluorescence microscopy (cells on coverslips)

1) Wash neurons rapidly, but carefully x2 with PBS, and replace the final wash with -20°C methanol. Incubate cells for 5 min. We store methanol at -20°C, and remove immediately prior to cell fixation. Fixation is carried out on the bench.
2) Wash fixed cells x2 with PBS (~5 min each wash) and proceed directly to primary antibody stage (no blocking is required).
3) Incubate coverslips with primary antibodies diluted in PBS for 2 hour (room temperature) or overnight at 4°C (depending on the antibody; see Table 4.1).
4) Wash coverslips x3 with PBS and proceed directly to secondary antibody stage.
5) Incubate with appropriate secondary antibodies at room temperature for 1 hour.
6) Wash x3 with PBS (5 min each).
7) Remove coverslips, then mount by inverting on to a 5μl drop of Mowiol placed on a standard glass slide. Press down carefully and leave to set for minimum 6 hours.

4.1.2 Paraformaldehyde fixation for immunofluorescence microscopy (cells on coverslips)

1) Wash neurons rapidly, but carefully with PBS.
2) Fix in 4% PFA for 20 min (enough to comfortably immerse the coverslip).

3) Permeabilisation and blocking is achieved by incubating neurons with blocking solution containing 5% BSA and 0.3% Triton X-100 in PBS for 30-60 min at room temperature.
4) Incubate coverslips with primary antibodies diluted in 50% blocking solution (2.5% BSA and 0.15% Triton X-100 in PBS) for 2 hourd (room temperature) or overnight at 4°C (depending on the antibody; see Table 4.1).
5) Wash coverslips x3 with PBS and proceed directly to secondary antibody stage.
6) Incubate with appropriate secondary antibodies diluted in 50% blocking solution at room temperature for 1 hour.
7) Wash x3 with PBS (5 min each).
8) Mount by inverting on to a 5μl drop of Mowiol placed on a standard glass slide. Press down carefully and leave to set for minimum 6 hours.

***Notes*:**

- Alternative blocking solutions comprising 10% normal donkey serum (for anti-TH staining), or 0.2% BSA with 0.2% Triton X-100, have also been tested with similar results.
- For lysosomal staining, 0.1% saponin should be used as permeabilisation reagent in place of Triton X-100 to maintain lysosomal structure.
- Volumes for PBS washes, blocking solutions and antibodies depend upon the volume of the plate/well configuration. We use 4-well plates routinely, and for these, a volume of 250-500μl is adequate for washes etc.

4.2 Live-cell imaging of autophagy dynamics in mDAN cultures

Neuronal architecture means that more careful consideration of the location of autophagosome assembly and of the maturation-coupled transportation kinetics of the autophagosome pool is often desirable. Live-cell imaging provides a direct means to measure these parameters, and a number of vital stains (Table 4.3) are available. We also use lentiviruses to express common autophagy reporters (e.g. GFP-LC3B (see [1]); mCherry-GFP-LC3B; ATG5-GFP (see [1]); mCherry-GFP-FIS1 (Fig. 3D) [15]).

1) Prepare live-cell imaging dishes by coating as described in Section 3.3.2.
2) Plate cells as described in Section 3.4.3.
3) For lentiviral transduction infect cells for 3 days then replace media with N2B27 supplemented with 20ng/ml BDNF, 20ng/ml GDNF, 0.2mM ascorbic acid, 500μM db-cAMP and 10μM DAPT. Incubate cells for another 2-4 days.
4) For staining with fluorescent probes, wash cells with PBS and then treat cells with the appropriate probes diluted in N2B27 supplemented with 20ng/ml BDNF, 20ng/ml GDNF, 0.2mM ascorbic acid, 500μM db-cAMP and 10μM DAPT for the indicated times at 37°C (see table 4.2.)

Wash cells twice with PBS then add fresh N2B27 supplemented with 20ng/ml BDNF, 20ng/ml GDNF, 0.2mM ascorbic acid, 500µM db-cAMP and 10µM DAPT.

Notes:

- Like primary neurons, hiPSC-derived neuronal cultures are fragile and are more prone to illumination damage than standard, undifferentiated laboratory cell-lines.
- Neuronal architecture dictates that the cell body (soma) may be some mm distance from growing neurite tips. This affects analysis of autophagosome assembly and intensity analyses.
- Successful hiPSC-derived neuronal cultures are complex, with multiple neurites intersecting one another on the plane of the coverslip. This makes it difficult to associate cell bodies with peripheral neuronal structures.
- Common promoters for expression of transgenes do not work with all hiPSC-derived neuronal types (e.g. the CMV promoter is silenced in midbrain neuronal cultures). For promoters, we use synapsin, EF1a, or spleen focus forming virus (SFFV) [16].
- For lentiviral transduction, neural progenitors are plated in live imaging dishes or on coverslips. Cells are infected for 3d, and then media is replaced with complete N2B27 and cells incubated for another 2-4d. These long incubation periods are needed to allow sufficient transgene expression.
- After a live imaging experiment, it is advisable to ensure that cells examined are confirmed mDANs. To do this, we stain cells post-imaging with anti-TH antibodies after PFA fixation as described in Section 4.1.2 (Fig. 3C). Using an automated microscope set up and image acquisition software such as MetaMorph (Universal Imaging) can be of assistance as it is possible to record field positions to allow multi-point time-lapse imaging with retrospective TH labelling.

5. Summary

The ability to monitor autophagy in disease-specific cell-types derived from hiPSCs provides researchers with the capacity to determine how autophagy contributes to neurodegenerative disease onset and progression, and the means to carry out functional screens in appropriate cell-line models [1, 2]. We have presented a protocol for the rapid generation of human mDANs from hiPSCs for PD-based research, and have outlined considerations for imaging based autophagy analysis. In theory, any imaging-based autophagy assessment approach; however, the unique architecture and fragility of hiPSC-derived neuronal cultures dictates that additional care is needed in the selection and application of assessment methodologies when using these cells.

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Figure legends

Figure. 1: Feeder-free HIPSC colony morphology during a complete cycle of seeding*, proliferation, enzymatic detachment and re-seeding.* (A) One day after initial plating. The colonies start forming, but cells within remain loosely connected and the colonies have non-uniform margins. (B) Day 2; the colonies have expanded and have started to adopt a tighter density, still with uneven margins. (C) Day 3; tighter colonies of cells are seen with smoother margins. (D) Day 4: colonies have become significantly larger, with more densely packed cells, and with smooth margins. The colonies are now ready to be passaged. (E) Chemical passaging with EDTA; 5 minutes after EDTA application, the cells in the colonies start to separate. They are detached by gently forcing with a 1ml pipette tip. (F) This image shows colonies that have detached, have been triturated into small clusters using a 1ml pipette tip, and have been passaged on to new vitronectin coated plates. The following day, the cells will once again look like those of image (A). Scale bars: 200μm.

Figure. 2: Adapted monolayer protocol for the differentiation of hiPSCs into mDANs. hiPSCs are cultured in E8 essential medium until reaching 70% confluency (days -4 to 0). Neuralisation starts at day 0 when the media is exchanged for N2B27 supplemented with neural fate inducing (SB and LDN) and patterning (SHH and CHIR) factors. Days 3 and 7 are the typical passaging days. At day 9 all of the above factors are removed and the cells are maintained for another 2 days in N2B27. At this point, media is replaced with N2B27 supplemented with neurotrophic factors (BDNF, GDNF and AA), and the cells are passaged again at day 12 into N2B27 with neurotrophic factors. From that point the cells are passaged once per week or when their confluency reaches the 80-85%. For terminal differentiation, from day 15 and onwards, the cells are administrated with db-cAMP and DAPT.

Figure. 3: Examples images of hiPSC neuronal cultures and autophagy/mitophagy assessments. (A) Examples of hiPSC-derived mDAN cultures stained with anti-TH antibodies in combination with anti-βIII tubulin (TUJ1; top) and FOXA2 (bottom) antibodies. Bar = 20μm. (B) Example field of hiPSC-derived cortical neurons stained with anti-βIII tubulin (TUJ1) and anti-TBR1. Bar = 20μm. (C) Example image of a TH-positive mDAN labelled using CytoID. Cells were treated for 4 hours with 1μm AZD8055, labelled with CytoID, then fixed and stained with anti-TH antibodies. Bar = 10μm. (D) Mitophagy assessment using the tandem mCherry-GFP-FIS1 reporter [15]. hiPSC-derived neuronal cultures were transduced with lentivirus expressing mCherry-GFP-FIS1 and imaged live before fixing and labelling with anti-TH antibodies. Arrows show red puncta indicative of mitochondria delivered to lysosomes. Arrowhead indicates the position of the soma. Bar = 10μm.

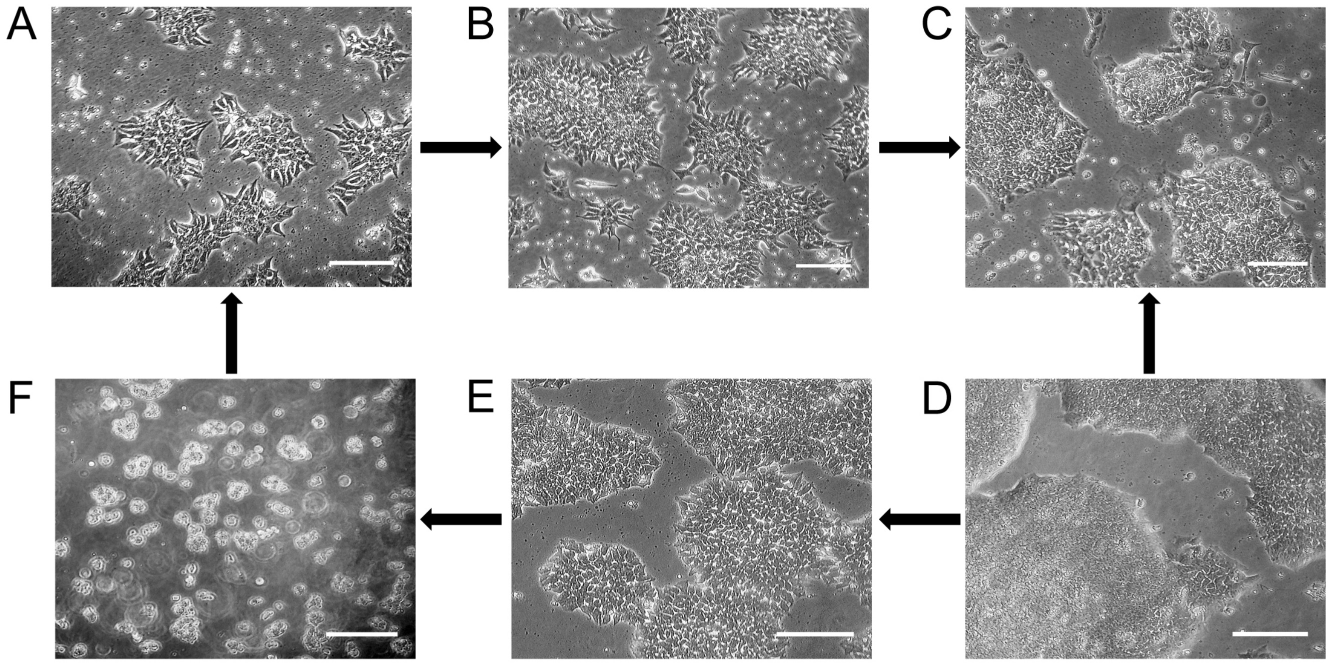

Fig. 1

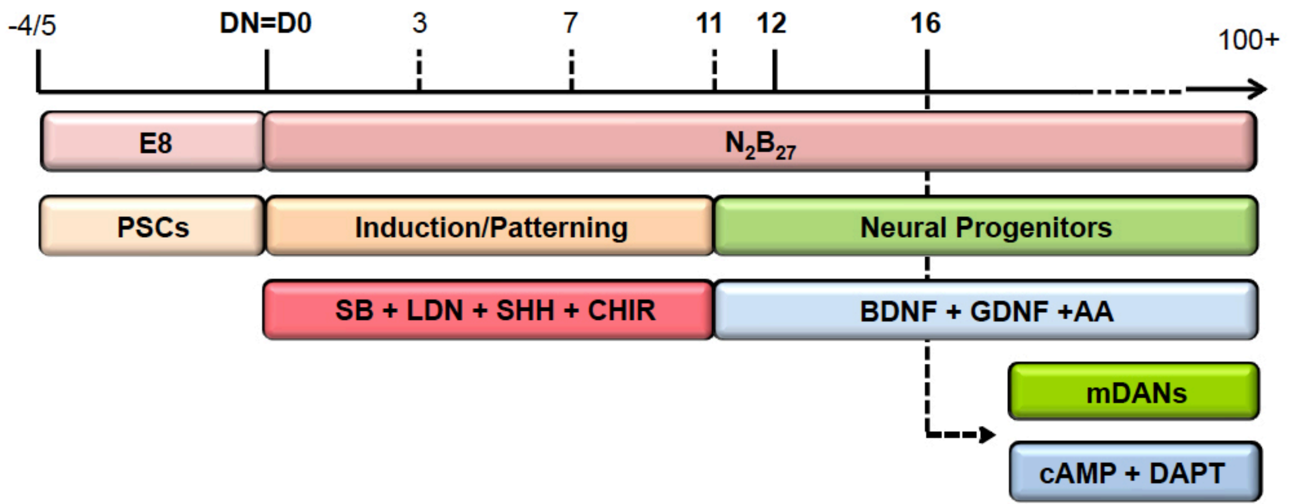

Fig. 2

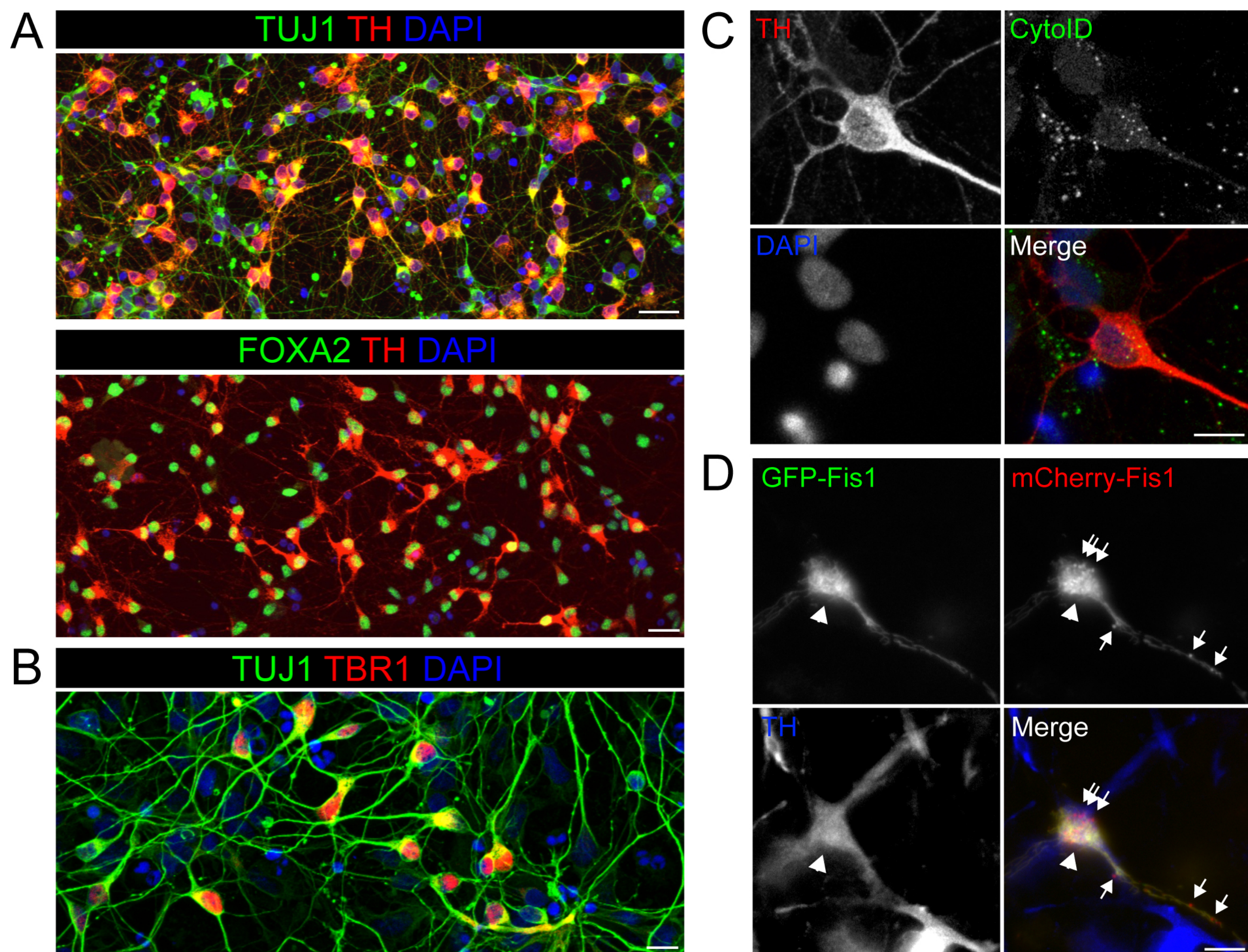

Fig. 3