Cultivation, differentiation, and lentiviral transduction of human induced pluripotent stem cell (hiPSC)-derived glutamatergic neurons for studying human TAU

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Method Article

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Abstract

TAU pathology is a major hallmark of many neurodegenerative diseases summarized under the term tauopathies. In most of these diseases, e.g., Alzheimer's Disease, the neuronal axonal microtubule-binding TAU protein becomes mislocalized to the somatodendritic compartment. In human disease, this missorting of TAU is accompanied by an abnormally high phosphorylation state of the TAU protein, and several downstream pathological consequences (e.g. loss of microtubules, degradation of postsynaptic spines, impaired synaptic transmission, neuronal death). While some mechanisms of TAU sorting, missorting and associated pathologies have been addressed in rodent models, few studies have addressed human TAU in physiological disease-relevant human neurons. Suitable human-derived *in vitro* models are necessary. This protocol provides a simple step-by-step protocol for generating homogeneous cultures of cortical glutamatergic neurons using an engineered *Ngn2* transgene carrying WTC11 iPSC line. We further demonstrate strategies to improve neuronal maturity, i.e., synapse formation, TAU isoform expression, and neuronal activity by co-culturing hiPSC-derived glutamatergic neurons with mouse-derived astrocytes. Finally, we explain a simple way for high-efficiency lentiviral transduction of hiPSC-derived neurons at almost all stages of differentiation.

Introduction

The microtubule-associated protein TAU is highly abundant in the axons of human brain neurons (Binder et al., 1985). By binding axonal microtubules (MTs) with its C-terminal MT-binding domain, TAU increases the stability and polymerization of MT filaments (reviewed in Barbier et al., 2019). Thereby, TAU controls essential axonal functions like elongation, branching, or cargo transport (reviewed in Arendt et al., 2016). Somatodendritic TAU accumulation is considered a pathological key event of many neurodegenerative diseases called tauopathies (reviewed in Zempel & Mandelkow, 2014). This TAU missorting coincides with an abnormally high phosphorylation state of TAU that decreases its MT binding affinity leading to elevated mobility of TAU. Missorted TAU can induce TTLL-mediated loss of dendritic MT filaments, resulting in degradation of postsynaptic spines, impaired synaptic transmission, and eventually neuronal dysfunction (Schützmann et al., 2021; Zempel et al., 2013; Zempel & Mandelkow, 2015).

Since TAU missorting is a critical step in the pathological cascade, numerous studies have focused on mechanisms that might underlie subcellular TAU sorting. Different reports suggested the relevance of either substantial somatic TAU degradation, active axonal TAU transport, axonal TAU retention, or even preferential axonal TAU translation (Zempel & Mandelkow, 2019). In addition, the axon initial segment (AIS) is thought to be critical for TAU enrichment as it regulates anterograde and retrograde TAU transit (Li et al., 2011; van Beuningen et al., 2015; Zempel et al., 2017). However, we still lack a conclusive model of how cellular components and TAU-intrinsic motifs collaborate during the sorting process and how this interplay becomes compromised during pathological missorting.

One model of choice for modeling TAU physiology and pathological functions *in vitro* are human-derived induced pluripotent stem cells (hiPSCs)-derived neurons. However, the differentiation of hiPSCs into

functional neurons often requires complex differentiation procedures that are time-consuming and often result in heterogeneous cultures, including different neuronal subtypes and considerable amounts of glial cells (Muratore et al., 2014; Nicholas et al., 2013).

In this protocol, we provide a simple step-by-step protocol for generating homogeneous cultures of cortical glutamatergic neurons using an engineered WTC11 iPSC line, which carries an inducible *Neurogenin2* (*Ngn2)* transgene in the AAVS1 gene locus (Hayashi et al., 2016; Kreitzer et al., 2013; Miyaoka et al., 2014; Wang et al., 2017). We further demonstrate strategies to improve neuronal maturity, i.e., synapse formation, TAU isoform expression, or neuronal activity by co-culturing hiPSC-derived glutamatergic neurons with mouse-derived astrocytes. Finally, we explain a simple way for high-efficiency lentiviral transduction of hiPSC-derived neurons at almost all stages of differentiation.

Reagents

1.1. Cell lines

· *Ngn2*-WTC11 hiPSCs as generated by . See **Note 1.**

Wang et al. (2017)

· HEK293T cells (ATCC, CRL-3216)

1.2. HiPSC maintenance reagents

· Geltrex LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix (Thermofisher Scientific, A1413302): Thaw Geltrex (GT) overnight at 4°C. All following steps must be carried out on ice with pre-cooled labware. Dilute 5 ml Geltrex with 5 ml ice-cold KnockOut DMEM, vortex to mix. Prepare 300 µl aliquots in 15 ml conical tubes and store at -20°C.

· KnockOut DMEM (Thermofisher Scientific, 10829018)

· StemMACS iPS-Brew XF (Miltenyi Biotec, 130-104-368): add 10 ml StemMACS iPS-Brew XF 50× Supplement to 500 ml media before usage.

· 10 mM Thiazovivin (Axon Med Chem, 1535): Dissolve 5 mg of Thiazovivin in 1605 µl DMSO, aliquot to 50 µl and store at -20°C.

· Dulbecco's Phosphate Buffered Saline (DPBS; Merck, D8537)

· Versene (Thermofisher Scientific, 15040066)

· KnockOut Serum Replacement (KSR, Thermofisher Scientific, 10828028)

· Antibiotic-Antimycotic Solution 100x (Merck, A5955)

1.3. HiPSC differentiation reagents

· Accutase (Merck, A6964)

· KO DMEM/F-12 (Thermofisher Scientific, 12660012)

· DMEM/F-12 (Thermofisher, 11320033)

· Neurobasal Medium (Thermofisher, 21103-049)

· GlutaMAX Supplement 100x (Thermofisher Scientific, 35050061)

· B27 Supplement, 50x, serum free (Thermofisher Scientific, 17504044)

· Neuropan 2 Supplement, 100x (Pan-Biotech, P07-11010)

· Non-essential amino acids (NEAA, Thermofisher Scientific, 11140035)

· 10 µg/ml Recombinant Human BDNF (Peprotech, 450-02): Dissolve 10 µg in 1 ml DPBS+0.1 % BSA, aliquot and store at -20 °C.

· 10 µg/ml Recombinant Human NT-3 (Peprotech, 450-03): Dissolve 10 µg in 1 ml DPBS+0.1 % BSA, aliquot and store at -20 °C.

· Laminin from Engelbreth-Holm-Swarm murine sarcoma basement membrane (Sigma, L2020): Laminin concentration is batch-dependent and must be considered when preparing pre-differentation or maturation medium for the differentiation of hiPSCs.

· 1 mg/ml Doxycycline hyclate (Merck, D9891): Dissolve 10 mg in 10 ml ddH_20, aliquot and store at -20 °C.

· Trypan Blue (BioRad, 1450021)

· 1 mg/ml Poly-D-Lysine (Merck, P7886): Dissolve 10 mg PDL in 10 ml DPBS. Store 1 ml aliquots at -20 °C.

· 6 mg/ml Cultrex 3D Culture Matrix Laminin I (Biotechne, 3446-005-01): Aliquot and store at -20 °C.

1.4. Primary glial cell culture reagents

· Hank's Balanced Salt Solution (HBSS) without Calcium and Magnesium (Thermofisher Scientific, 14175095)

· Trypsin 0.5 %/EDTA 0.2 % in PBS, w/o: Ca and Mg (10x, Pan-Biotech, P10-024100)

· Fetal Bovine Serum (FBS, Biochrom AG, S 0615)

· DMEM/ F-12 with GlutaMAX supplement (Thermofisher Scientific, 10565-018)

· 1 mg/ml Cytosine β-D-arabinofuranoside (AraC , Merck, C1768): Dissolve 10 mg in 1 ml ddH_20, filter sterile, aliquot and store at -20 °C.

· Dimethylsulfoxid (DMSO, Carl Roth, A994)

1.5. HEK293T maintenance reagents

· Trypsin 0.5 %/EDTA 0.2 % in PBS, w/o: Ca and Mg (10x, Pan-Biotech, P10-024100)

· DMEM (Thermofisher Scientific, 31966047)

· Fetal Bovine Serum (FBS, Biochrom AG, S 0615)

· Antibiotic-Antimycotic Solution 100x (Merck, A5955)

1.6. Transduction reagents

· Polyethylenimine (PEI) transfection reagent (#23966, Polysciences): prepare according to manufacturer's protocol.

· Lentiviral plasmids (**see Note 2**):

o Transfer plasmid, e.g., pUltra (Addgene #24129)

o envelope plasmid, e.g., pMD2.G (Addgene #12259)

o packaging plasmid, e.g., psPAX2 (Addgene #12260)

· DMEM (without supplements, serum and antibiotic-free)

1.7. Medium recipes

· iPSC- freezing medium: Mix 9 ml KSR with 3 ml Brew and 3 ml DMSO, store at 4 °C for up to one month.

· cBrew: StemMACS™ iPS-Brew XF with supplement, add 1x Antibiotic- Antimycotic Solution

· Pre-differentiation medium:

o KO DMEM/F-12

- 1x Neuropan-2 Supplement
- 1x non-essential amino acids
- 10 ng/ml BDNF
- 10 ng/ml NT-3
- 1.5 µg/ml Laminin
- 2 µg/ml Doxycycline
- 1x Antibiotic- Antimycotic solution

· Neuronal maturation medium

- 50 % Neurobasal Medium
- 50 % DMEM/F-12
- 0.5x Neuropan-2 supplement
- 1x non-essential amino acids
- 0.5x GlutaMax
- 0.5x B27 Supplement
- 10 ng/ml BDNF
- 10 ng/ml NT-3
- 1.5 µg/ml Laminin
- 2 µg/ml Doxycycline
- 1x Antibiotic- Antimycotic solution

· Astrocyte maintenance medium (AMM):

- DMEM/F-12 with GlutaMAX
- 10 % FBS
- 1x Antibiotic- Antimycotic Solution

· Astrocyte freezing medium:

- 90 % FBS
- 10 % DMSO

· HEK maintenance medium:

- DMEM, high glucose, GlutaMAX Supplement, pyruvate (Thermofisher Scientific, 31966047)
- 10 % FBS
- 1x Antibiotic- Antimycotic Solution

Equipment

Procedure

1.1. Cultivation of hiPSCs

1.1.1. Coating of culture plates

For routine cultivation of hiPSCs, cell culture plates are coated with Geltrex (GT). Coating on the day of usage is highly recommended, and long-term storage of plates might influence cell survival.

1. Thaw one aliquot (300 µl) of GT at 4 °C.
2. Dilute with 14 ml KnockOut DMEM (KO DMEM) to working concentration (~200 µg/ml).
3. Add diluted GT to cell culture plates (i.e., 1 ml for a well of a 6-well plate) and incubate at least for 30 min at 37 °C.
4. Plates are ready to use; no further washing is required before seeding of cells.

1.1.2. Thawing of hiPSCs

Coat two wells of a 6-well plate using GT as described before (see **3.1.1.**).

1. Pre-warm all necessary reagents to 37 °C using a water bath.
2. Prepare 10 ml of KO DMEM in a 15 ml conical tube.
3. Thaw one vial of *Ngn2*-WTC11 iPSCs for 1-2 min in a 37 °C water bath. Rapidly proceed with the following steps to avoid extensive exposure of cells to DMSO.
4. Transfer vial to a sterile clean bench and pour cell suspension to KO DMEM containing conical tube.

5. Invert cells carefully 2-3 times.

6. Centrifuge cells at 400g for 5 min.

7. Carefully aspirate supernatant and resuspend cells in 5 ml cBrew. It is essential to keep smaller cell clumps and not completely dissociate cells. This increases survival and avoids spontaneous differentiation.

8. Add 1 µl 10 mM Thiazovivin (T; 1:5000 dilution, final concentration: 2 µM) to the cell suspension.

9. Aspirate GT from culture plates and seed 2.5 ml of cell suspension to each well.

10. On the next day: change media to fresh cBrew without T (see **Note 3**).

1.1.3. Routine cultivation of hiPSCs

For the routine maintenance of hiPSCs, media should be exchanged every 2-3 days. Passage cells when they reach ~70-80 % confluency. Monitor cells under a microscope and remove all spontaneous differentiating cells before continuing (see **Note 4**). Healthy cultures of undifferentiated hiPSCs are shown in **Figure 1**.

Remove differentiating cells from the cultures before passaging.

1. Coat the desired amount of cell culture wells using GT as described before.

2. Pre-warm all necessary reagents to 37 °C using a water bath.

3. Wash cells once with DPBS.

4. For a 6-well, add 1 ml of Versene and incubate cells for 3-5 min at room temperature (RT) until cells start to dissociate.

5. Aspirate Versene carefully and add 1 ml cBrew.

6. Gently detach colonies using a cell scraper.

7. Collect the cell-containing medium in a conical tube. Optional: add another 1 ml of cBrew to wells to collect remaining cells. Break up bigger clumps of hiPSCs by gentle trituration with a 5 ml plastic pasteur pipette (see **Note 5**).

8. Dilute cells to desired density using cBrew. Routine passaging ratios are 1:5-1:20 (see **Note 6**)

9. Add 1:5000 Thiazovivin to cells and distribute cells to culture wells.

10. Change media the next day to fresh cBrew without Thiazovivin.

1.1.4. Freezing of hiPSCs

A confluency of about 70-80 % is recommended before freezing down the cells.

1. Prepare freezing medium and freshly add 1:2500 Thiazovivin (final concentration: 4 µM)

2. Follow steps 2. - 7. of **3.1.3.**

3. Collect 900 µl of cell suspension (avoid extensive pipetting to preserve cell clumps) and add them to a cryovial.

4. Add 900 µl of iPSC-freezing medium directly to the cells. Proceed as fast as possible to avoid extensive exposure of cells to DMSO.

5. Transfer cells to a freezing device (Mr. Frosty) and store them at -80 °C for short-term storage (less than a week). For long-term storage, transfer cells to -180 °C.

1.2. Doxycycline-induced differentiation of hiPSCs into cortical glutamatergic neurons

1.2.1. Pre-differentiation

Cultivate hiPSCs on GT-coated plates until they reach around 70-80 % confluency. Prepare fresh GT-coated 6-well plates. Pre-differentiation is started 3 days before sub-plating and start of differentiation (day -3) (see **3.2.3.**).

1. Pre-warm all necessary reagents to 37 °C using a water bath.

2. Wash cells once with DPBS

3. Add 1 ml accutase to each well, incubate 5- 8 min at 37 °C, until all cells are detached from the culture plate; non-detached cells can be detached by gently tapping the culture plate.

4. Stop accutase treatment by adding 3 ml DPBS to the cells.

5. Collect cells in a 15 ml conical tube, centrifuge at 400 g for 5 min, discard supernatant carefully with a vacuum pump.

6. Resuspend cells in 0.5 - 1 ml cBrew depending on pellet size.

7. Count viable cells using an automated cell counter and trypan blue.

8. Dilute to desired density using pre-differentiation medium with 1:5000 Thiazovivin

9. Seed 1.5 - 2 x 10^6 cells to each well of a 6-well plate in 3 ml.

10. Day -2 and -1: Exchange media to fresh pre-differentiation medium without Thiazovivin. Since cells have high density use at least 3-4 ml.

Pre-differentiated hiPSCs at day 0 (before seeding) are shown in **Figure 1**.

1.2.2. Coating of culture plates for hiPSC differentiation

Prepare coatings 1 day before starting the differentiation (see **Note 7**). Thaw PDL and Cultrex 3D Laminin aliquots at 4 °C. For imaging of neurons, grow the cells on glass coverslips.

1. Prepare 20 µg/ml PDL: dilute 1 ml aliquot of 1 mg/ml PDL in 50 ml DPBS.
2. Add PDL to culture plates and incubate overnight at 37 °C (see **Note 8**).
3. On the following day: Thaw Cultrex 3D Laminin aliquot at 4 °C.
4. Remove PDL from plates and wash once with DPBS.
5. Dilute Cultrex 3D Laminin to 20 µg/ml with DPBS.
6. Add Laminin to culture plates and incubate for at least 1 hour at 37°C.
7. Wash twice with DPBS before seeding of cells.

1.2.3. Differentiation of hiPSCs into cortical glutamatergic neurons

Start differentiation at day 3 after pre-differentiation (day 0, **see Figure 1**):

1. Follow steps 1. – 5. of **3.2.1.**
2. After centrifugation, resuspend cells in 1 ml maturation medium.
3. Count viable cells using an automated cell counter and trypan blue.
4. Dilute cells to desired density using maturation medium containing freshly added 1:100 GT
5. Recommended seeding densities:

a. 24-well plate: 50. – 80.000 cells/well

b. 6-well plate: 250. – 400.000 cells/well

6. If monocultures are performed, exchange half of the maturation medium every week until further analysis.

Differentiated hiPSC-derived neuron cultures are shown at different stages of differentiation in **Figure 1**.

1.2.4. Co-cultivation with primary mouse glial cells

Co-cultivation of hiPSC-derived neurons with primary glial cells is beneficial for synapse formation and neuronal activity of hiPSC-derived neurons . In addition, we could previously show that co-cultivation increases neuronal activity, assayed by calcium-oscillation based live-imaging (for details see .

(Johnson et al., 2007; Pang et al., 2011; Vierbuchen et al., 2010; Zhang et al., 2013)

Bachmann, Linde, et al., 2021)

1.2.4.1. Isolation of glial cells from embryonic mouse brain

Glial cells are isolated from WT mice at embryonic day E13.5; brain stem and cortical hemispheres can be used for glial cell isolation. Before the start of preparation, sterilize all tools with ethanol. Work on ice during the preparation of the brain and use only pre-cooled reagents and tubes. Prepare PDL-coated T-75 flasks as described above (see **3.2.2**).

Note: Animal experiments require authorization by the responsible supervising authority. All our animal experiments are approved by the Animal Welfare Officer of the University of Cologne (according to §4 TierSchG, Germany).

1. Euthanize a pregnant mouse and dissect the heads of E13.5 embryos.

2. Remove scalp and non-brain tissue from the head to dissect the brain in ice-cold HBSS + 1X Antibiotic-Antimycotic solution.

3. Carefully but thoroughly remove meninges from the brain

4. Divide brainstem and cortical hemispheres.

5. Transfer brain parts into a reagent tube containing ice-cold HBSS+1x Antibiotic-Antimycotic solution and keep on ice.

6. Pool 2-3 brains to yield sufficient glial cells.

7. Transfer to a sterile hood for cell isolation.

8. Manually wash brain pieces twice with HBSS + 1x Antibiotic-Antimycotic solution

9. Add trypsin to brains and incubate for 7 min at 37 °C.

10. Stop reaction by adding HBSS+10 % FBS

11. Discard the supernatant and dilute cells in HBSS + 1x Antibiotic-Antimycotic solution

12. Dissolve tissue by trituration using a P1000 pipette.

13. Count viable cells with an automated cell counter and trypan blue.

14. Dilute cells in Astrocyte maintenance medium (AMM) and seed onto T-75 flasks.

1.2.4.2. Maintenance & freezing of glial cells

Monitor glial cells regularly. Depending on confluency, exchange media once or twice a week. Routinely passage glial cells using trypsin when they reach >80 % confluency (see **Note 9**). Glial cells can be cryopreserved in 90 % FBS+ 10 % DMSO.

1.2.4.3. Seeding of primary mouse glial cells to hiPSC-derived neurons

For co-cultivation of hiPSCs-derived neurons and primary glial cells, a 1:1 to 2:1 ratio is favored. This will result in higher synapse count and increased neuronal activity as assayed by calcium imaging (see **3.2.4**). The addition of glial cells to neurons is ideally performed between days 2 to 4 after neuronal differentiation. Plan the isolation and cultivation of primary glial cells accordingly to yield sufficient cell counts.

1. Wash glial cells once with DPBS.

2. Add trypsin and incubate cells for 5 min at 37 °C.

3. Add AMM to stop the reaction and collect cells in a 15 ml conical tube.

4. Centrifuge cells at 400 g, 5 min.

5. Resuspend in 1 ml DMEM/F-12 (without supplements).

6. Count viable cells with an automated cell counter and trypan blue.

7. Add glial cell suspension dropwise to hiPSC-derived neurons, e.g., for a 6-well plate containing 2.5 x 10^5 neurons, add at least 1.25 - 2.5 x 10^5 glial cells.

8. On day 4: treat co-cultures with 4 µg/ml AraC. For this, exchange full maturation medium.

9. From day 5 to 9: exchange half of the medium twice a week.

10. From day 10: exchange half of the medium twice a week and add 2.5 % FBS to improve the survival of glial cells.

1.3. Lentiviral transduction of hiPSC-derived neurons

For assembling lentiviruses, multiple different vectors are required. The transfer plasmid carries the cDNA of the gene(s) of interest integrated into the host genome for stable protein expression. The transgene is flanked by long terminal repeat (LTR) sequences, which enable the integration of the transfer plasmid

elements into the host genome.
We achieved solid and reliable transduction efficiency for the third-generation transfer plasmid pUltra (Addgene #24129), based on the human immunodeficiency virus 1 (HIV1). The pUltra vector and its derivatives enable multicistronic expression of up to three genes of interest. The empty backbone contains an enhanced GFP (eGFP) with two downstream multiple cloning sites (MCS) in-frame. The eGFP and the MCSs are separated by two 2A peptide sequences. The 2A peptides induce cleavage of the protein chain after translation. This posttranslational severing ensures equal expression of eGFP and the downstream proteins. Publicly available pUltra derivatives contain dTomato instead of eGFP (pUltra-Chili) or harbor a tetracycline-responsible element (TRE), allowing inducible gene expression (pUltra-dox). Other available transfer plasmids, e.g., the IRES-based pWPI or the pLKO knockdown vectors, may also be feasible for the transduction of hiPSC-derived neurons. Using pUltra, we regularly achieve a >90 % transduction efficiency using lentiviral transduction even of very mature (30+ DIV) iPSC-derived neurons.

The packaging and envelope plasmids encode for viral proteins Gag, Pol and VSV-G, which are essential for the lentivirus assembly. Here, we used the vectors psPAX2 (Addgene ##12260) and pMD2.G (Addgene #12259), respectively. Other second-and third-generation packaging plasmids might be feasible as well.

1.3.1. HEK293T maintenance

Grow HEK293T in HEK maintenance media on T-75 flasks and monitor regularly. Depending on confluency, exchange media once a week. Routinely passage HEK293T cells using trypsin when they reach >80 % confluency (we use a 1:10 passaging rate; however, this can be up- or downscaled accordingly). HEK293T cells can be cryopreserved in 90 % FBS + 10 % DMSO.

1.3.2. Virus particle production

Seed cells 1 day before transfection of viral plasmids (Day -1):

1. Seed HEK293T cells in a T-75 flask with a density that the cells reach 60-80 % confluency on day 0.

Continue with transfection on day 0:

2. Prepare the following transfection mixture:

a. 10 µg of transfer plasmid (pUltra + gene of interest)

b. 9 µg of envelope plasmid (pMD2.G)

c. 1 µg of packaging plasmid (psPAX2)

d. 20 µl of PEI

Fill up to 400 µl with DMEM (free of serum and antibiotics)

3. Mix gently (without vortexing) and incubate for 20 minutes at room temperature.

4. Wash the HEK293T cells with PBS, then add 5 ml of fresh HEK maintenance medium.

5. Add the transfection solution very slowly and dropwise onto the cells.

Day 1 (Medium addition):

6. Add 3 ml of HEK maintenance medium to the cells.

Day 2 (Medium change):

7. Replace the medium with 8 ml fresh HEK maintenance medium.

Day 3 (Virus harvesting I):

8. Collect the whole cell culture supernatant in a 15 ml falcon.

a. Add 8 ml of fresh HEK maintenance medium for harvesting on the following day.

9. Centrifuge the collected medium for 4 minutes at 400g to remove remaining HEK293T cells.

10. Filter the virus-containing supernatant using a 0.45 µm filter and a syringe.

11. Aliquot the solution into microcentrifuge tubes (~ 1 ml per aliquot) and place on ice.

12. Store the virus aliquots at -80 °C until further use.

Day 4 (Virus harvesting II):

13. Collect the medium in a 15 ml falcon.

a. Discard the HEK293T culture

14. Centrifuge the collected medium for 4 minutes at 400 g to remove remaining HEK293T cells.

15. Filter the virus-containing supernatant using a 0.45 µm filter.

16. Aliquot the solution into microcentrifuge tubes (~ 1 ml per aliquot) and place on ice.

17. Store the virus aliquots at -80 °C until use.

1.3.3. Transduction of hiPSC-derived neurons

All medium changes must be done as fast as possible and without any larger delay. Avoid the hiPSC-derived neurons being exposed to air.

Day 0 (Transduction):

1. Pre-warm neuronal maturation medium (at least room temperature, better 37 °C).

2. Thaw virus particles either slowly at 4 °C or rapidly using the water bath (see **Note 10**).

3. Prepare for each 24-well 250 µl (6-well: 1.0 ml) of total medium

a. Mix virus solution and maturation medium to achieve the desired dilution (see **Note 11 and 12**).

4. Put the virus aliquot(s) back to -80 °C as soon as possible.

5. Aspirate the medium from the cultures and store it in a separate tube.

6. Immediately add the virus mixture and maturation medium to the hiPSC-derived neurons (see **Note 13**).

Day 1 (Transduction stop):

7. Pre-warm the stored conditioned medium from day 0 (at least room temperature, better 37 °C).

8. Remove the virus-containing medium.

9. Optional: wash cells with pre-warmed DMEM-F12.

10. Add the pre-warmed conditioned medium.

11. Allow gene expression for the desired time (see **Note 14**).

In **Figure 2**, hiPSC-derived neurons are shown 6 days after transduction with pUltra-containing lentiviruses, using different virus dilution mixtures.

Troubleshooting

1. It is possible to use another commercially available cell line, e.g., BIONi010-C-15 (Bioneer; Schmid et al., 2021) or iP11N (Alstem), that carries the doxycycline-inducible *Ngn2* transgene. Alternatively, it is also possible to generate transgenic iPSCs with a doxycycline-inducible *Ngn2* by lentiviral transduction, see, e.g., Zhang et al., 2013; the lentiviral plasmid is deposited at Addgene #52047). HiPSC cell lines can be purchased, e.g., at Coriell Institute (Camden, NJ, USA). We have not tested other cell lines. Handling and differentiation of iPSCs and the maintenance of iPSC-derived neurons have to be adapted for the corresponding cell line.

2. The mentioned packaging plasmid belongs to the group of second-generation lentiviral vectors. Second-generation packaging plasmids can be combined with third-generation transfer plasmids such as pUltra. Third-generation packaging vectors, which provide higher safety precautions due to the absence of the viral Tat gene, are publicly available e.g.: pMDLg/pRRE (Addgene, #12251) and pRSV-Rev (Addgene, #12253).

3. Avoid incubating your iPSCs longer than 24 hours in media containing Thiazovivin. Extended exposure may result in substantial cytotoxicity.

4. Spontaneous differentiation sometimes occurs in hiPSCs cultures. To prevent extensive differentiation, remove differentiated cells using a microscope under a sterile hood. Simply aspirate cells using a P200 pipette. Alternatively, mark areas of spontaneous differentiation from the bottom of the plate and remove the cells by using a pipette tip (e.g., 10 µl size) connected to a vacuum pump.

5. For the passaging of hiPSCs with Versene, it is essential to preserve cell clumps at a specific size, too big or too small clumps can cause unwanted differentiation and extensive cell death; this size has to be estimated and optimized for the corresponding cell line.

6. Passaging ratios depend on experimental plans and confluency of the initial cultures, e.g., when passaging cells at 80 % confluency at a ratio of 1:10, cells reach confluency (~80 %) again after 3-4 days in our hands.

7. GT coating for at least half an hour (as described in **3.2.2.**) can be used as an alternative for PDL/ 3D Laminin to differentiate hiPSCs. Long-term cultivation (>6 weeks), however, might be affected.

8. Minimal coating time with PDL is 2 hours.

9. To avoid extensive growth of epithelial cells in primary glial cell cultures, cells can be shortly treated with trypsin (<3 min) to collect only epithelial cells and keep glial cells attached to the culture flask.

10. There is no clear evidence of whether lentivirus stability depends on the velocity of the thawing process. It is, however, generally recommended to thaw lentiviruses on ice since they might be sensitive to extreme temperature shifts (Jiang et al., 2015).

11. Repetitive thawing of virus aliquots reduces the yield of transduced neurons down to 50 % of the original level (Jiang et al., 2015). Therefore, this effect should be considered when determining an appropriate dilution factor.

12. The protein expression decreases with the number of introduced genes. The GFP expression appears stronger in neurons transduced with the empty pUltra backbone compared to pUltra for co-expression of GFP and another protein (see **Figure 2A,B**). Therefore, the optimal dilution factor must be determined separately for each virus particle.

13. Lentiviral transduction can be performed for hiPSC-derived neurons of all ages starting at day 7 of differentiation. Transduction might be feasible earlier but can increase the risk of substantial cell loss. To decrease cellular stress induced by the medium exchange, the virus solution can be directly added to the cell medium (adjust the medium amount before the desired dilution factor).

14. Please note that the rate of exogenous protein expression decelerates with the increasing age of the neuronal cultures. While for younger neurons (~ 2 weeks old), the GFP signal of empty pUltra can be

visible after 1-2 days, for older neurons (about 6-8 weeks old), this may take up to 1 week for similar virus titers.

Time Taken

Anticipated Results

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Figures

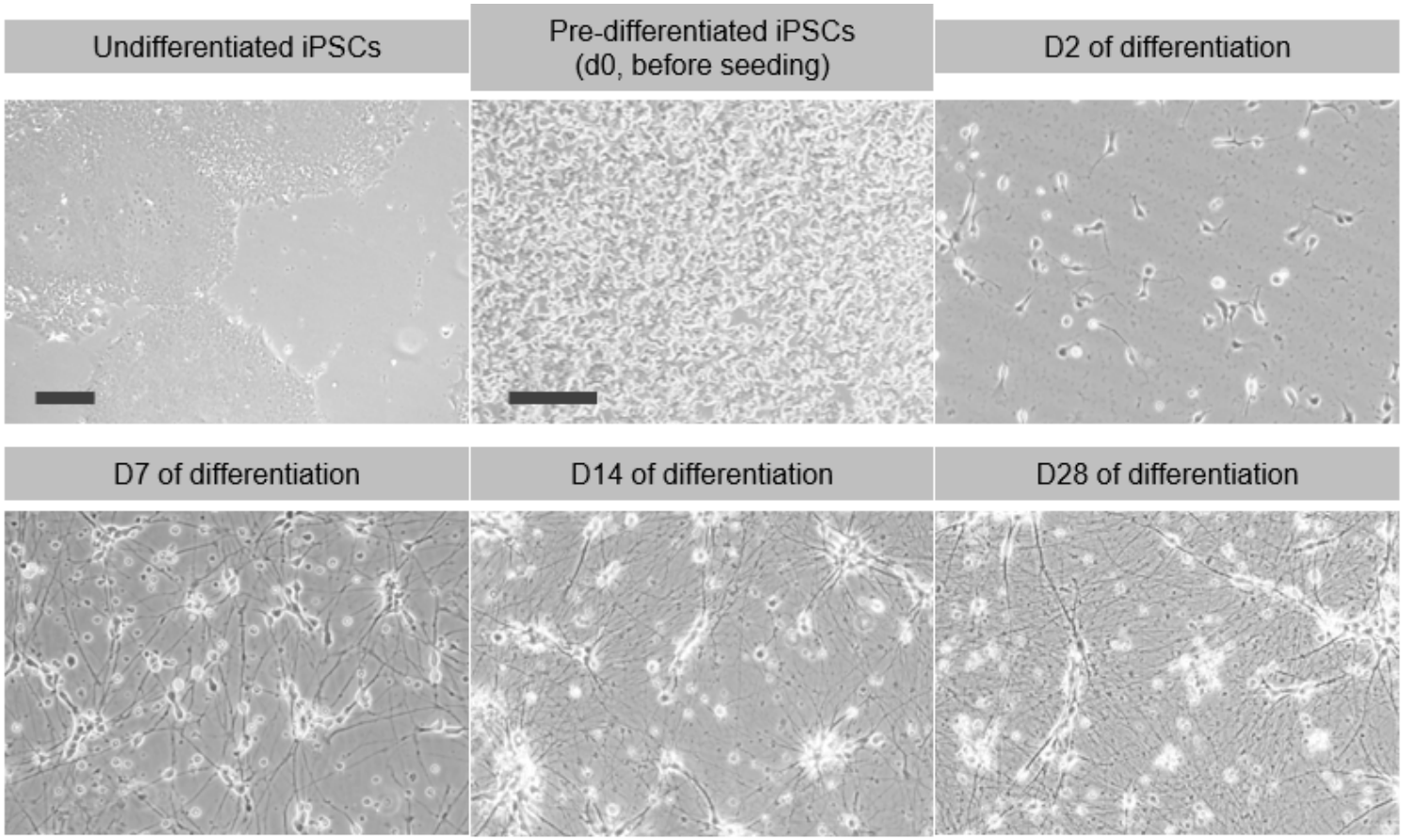

Figure 1

Neuronal differentiation of human WTC11 *Ngn2*-transgenic iPSCs.
Representative brightfield images of undifferentiated WTC11 *Ngn2*-transgenic iPSCs and different differentiation stages. Before differentiation, iPSCs typically grow in colonies (left top panel). For pre-differentiation, cells are seeded at high density. Addition of doxycycline leads to changed morphology and growth (middle top panel). Differentiated cells show growth of neurites already after two days of differentiation (right top panel). During differentiation, cells show extensive neuritic outgrowth and form a neuronal network (lower panels). See **3.2.** for the differentiation protocol. Scale bar (left top): 300 µm, scale bar (middle top): 150 µm. The latter scale bar corresponds to all other images during differentiation.

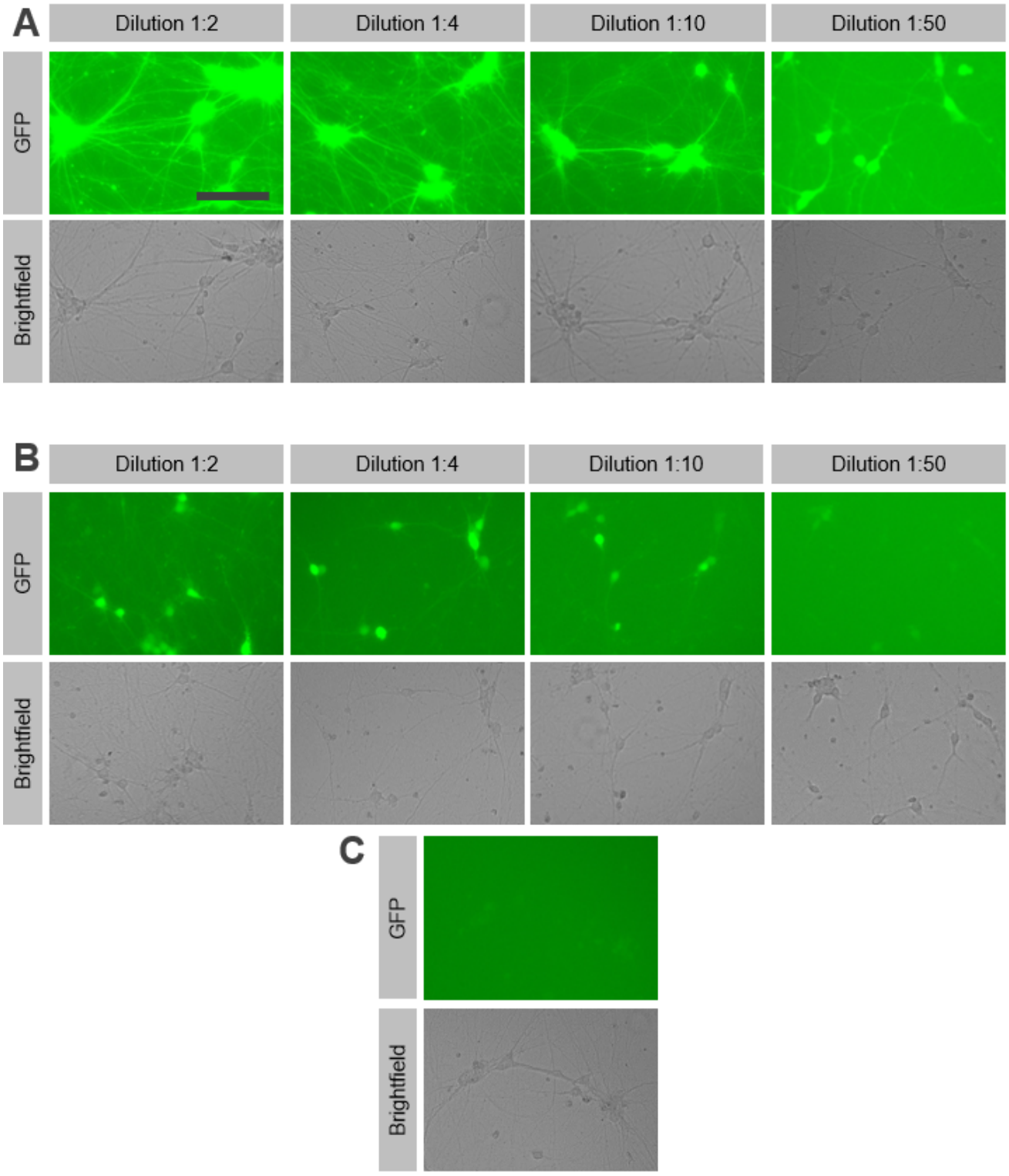

Figure 2

Lentiviral transduction of hiPSC-derived glutamatergic neurons.

Representative brightfield and fluorescence images of hiPSC-derived neurons after lentiviral transduction. The iPSC-derived neurons were transduced with virus particles containing either an empty pUltra vehicle plasmid (A) or pUltra carrying a BirA-0N3R-Tau fusion construct (B) at day 12 of differentiation. Four different dilutions of virus particles with culture medium (see protocol for details) were used, respectively.

Control cultures were not transduced at all (C). All cultures were imaged at day 18 of differentiation. Scale bar: 100 μm.