**The differentiation of GABAergic neurons**

1. In the differentiations, Matrigel coated plates were used. Matrigel was diluted 1/300 in DMEM/F12.

Firstly, you have to thaw Matrigel on ice and put on cold room 2-3 hours before coating.

For one well in 24-well plate🡪 0,5 ml

For one well in 6-well plate🡪 1 ml

For one 10cm dish🡪 5 ml

After adding matrigel to DMEM/F12, rotate the falcon gently (dont make any bubbles) about 20 times. Then put the plates or dishes inside the incubator. They have to stay inside of incubator at least 4 hours. I usually coat around 5pm and stayed them overnight.

1. Seeding cells for differentiation, wells were washed with PBS and 500μl Accutase per well was added. After 6 minutes incubation, cells were collected in the wells with PBS.
2. Cells were centrifuged at 300g for 4 minutes and supernatant was discarded. Cells were re-suspended with mTesR containing 1/1000 ROCKi.
3. Four wells from 6-well plate were seeded into 10cm dish (approximately 390 000 cells/cm2) Total media per dish is 8 ml with ROCKi
4. Next day, Dual SMAD inhibition was initiated by replacing medium with N3 media (Table 1) with SMAD inhibitors (Shi et al., 2012).

**Table 1: N3 media ingredients**

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Per 250 ml bottle** | **Company** | **Catalogue number** |
| DMEM/F12 + GlutaMAX Media | 120,5 ml | Gibco | 10565018 |
| Neurobasal Media | 120,5 ml | Gibco | 21103049 |
| B27 with Vitamin A (50X) | 2,5 ml | Gibco | 17504044 |
| Penicillin-Streptomycin | 2,5 ml | Gibco | 10378016 |
| N2 Supplement (100X) | 1,25 ml | Gibco | A1370701 |
| Non-essential amino acid | 1,25 ml | Gibco | 11140050 |
| GlutaMAX | 1,25 ml | Gibco | 35050061 |
| 2-mercaptoethanol (50 mM stock) | 250 μl | Gibco | 31350010 |
| Insulin (10mg/ml stock) | 62,5 μl | Sigma | 19278 |

SMAD inhibitors (add at time of use)

**Dorsomorphin (Sigma Aldrich, P5499-5MG):** Use 1:5000 (1μM final) (Martina is preparing, location is in the corner fridge in iPSC lab at -20 oC) It is stable at +4oC up to 7days.

**SB431542(Axon Medchem, 1661):** Use 1:1000 (10μM final) (Martina is preparing, location is in the corner fridge in iPSC lab at -20 oC) It is stable at +4oC up to 7days.

The addition to SMADi, WNT inhibitor 2μM XAV939 (Sigma Aldrich, X3004) was added (Close et al., 2017).

**XAV939 (Sigma Aldrich, X3004):** stock concentration is 5mg/ml in DMSO. (The location is in the corner fridge in iPSC lab at -20 oC) It is stable at +4oC up to 10days. It is stable at -20oC up to 6 months

1. The media was changed every day until day 10. (8ml per dish, you can increase the volume to 9ml when you see the media extremely yellow on the next day)
2. After day 10, protocol in Close et al., 2017 were followed. At day 10, 1 000 000 cells per cm2 were seeded onto Matrigel coated 6-well plates.
3. At day 11, ventral induction was started by replacing medium with N2 media (Table 2) with murine Shh 100ng/ml (5nM) (Peprotech, 315-22), 1μM XAV939 and PMA (0.5μM) (Sigma Aldrich, SML0868). Media was changed every two days from day 11 to day 17 (2ml per one well in 6-well plate). N2 media without Shh and PMA was changed from day 19 to day 23 (2ml per one well in 6-well plate).

**For these small molecules avoid repeated freze-thaw cycles. Prepare small aliqouts and use them twice (no more than three times).**

**Shh (Peprotech, 315-22)** stock concentration is 0,2mg/ml in PBS (and 0.1% BSA) For example: For 100ug Shh, reconstitute in 500 ul water + 0,5 ul BSA. It is stable at -80oC up to 1 year. It is in -80 (left one, in the bilgesu drawer) together with PMA.

**PMA (Sigma Aldrich, SML0868):** stock concentration is (9,6mM) 5mg/ml in DMSO. It is stable at -20oC up to 1 year. It is in -80 (left one, in the bilgesu drawer) together with Shh.

Dextrose is in the room where the ice box is. You have to weigh it outside. Therefore it has to be filtered.

**Table 2: N2 media ingredients**

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Per 250 ml bottle** | **Company** | **Catalogue number** |
| DMEM/F12 (1:1) | 240 ml | Gibco | 11320033 |
| 1X B-27 supplement (stock: 50X) | 5 ml | Gibco | 17504044 |
| 1X N-2 supplement (stock: 100X) | 2,5 ml | Gibco | A1370701 |
| 1X Penicillin-Streptomycin (stock: 100X) | 2,5 ml | Gibco | 10378016 |
| 55 μM 2-Mercaptoethanol (stock: 50mM) | 275 μl | Gibco | 31350010 |
| 0,15 % (w/v) dextrose | 0,375 gr | Sigma | D9434 |

1. At day 24, 800 000 cells per cm2 were seeded onto Poly-D-lysine (PDL)/Laminin coated plates.
2. PDL (stock concentration is 1mg/ml) is diluted in 1/50 in PBS and coated for overnight.

PDL is in Tatiana’s cell culture. When you enter it is on the left at small -20. 2nd shelf, clear white box.

Thaw PDL at water bath for 2mins before you add into PBS, mixed frequently. You can put remaining PDL at +4 oC afterwards and can stay there for one week.

- coat overnight hours (min 4 hours) at 37oC

For one well in 24-well plate🡪 0,5 ml

For one well in 6-well plate🡪 1 ml

- remove the liquid, wash 2-3x with water  
- dry inside the hood

After wells were dried, laminin (1/200 in PBS) coating is performed

**Important!** Final concentration of laminin should be 10ug/ml. Before using laminin check the lot number and protein content. It could ve sometimes lower and that can change the dilution. For example if protein content is 1.1 then dilution is 1/110.

Usually, I coat PDL and stayed overnight. In the morning, I coat laminin and do the splitting around 2-3pm.

1. For neuronal differentiation, several factors (Sodium-L-Ascorbate, Adenosine 3′,5′-cyclic monophosphate (cAMP), Neurotrophin-3 (NT-3), Brain-derived neurotrophic factor (BDNF) and Glial cell line-derived neurotrophic factor (GDNF)) were added into NBND media (Table 3) from day 25 (2ml per one well in 6-well plate). **Sodium-L-Ascorbate should be added daily.** The half of the medium was replaced every two days (1ml per one well in 6-well plate). At day 32, 200 000 cells per cm2 were plated onto PDL/Laminin coated plates. At day 33, medium was replaced with fresh NBND media (2ml per one well in 6-well plate).

**Table 4: NBND media ingredients**

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Per 250 ml bottle** | **Company** | **Catalogue number** |
| Neurobasal medium | 58 ml | Gibco | 21103049 |
| 0,5× B-27 supplement (stock: 50X) | 2,5 ml | Gibco | 17504044 |
| 2mM (1X) Glutamax Supplement (stock:100X, 200mM) | 2,5 ml | Gibco | 35050061 |
| 1X Penicillin-Streptomycin (stock: 100X) | 2,5 ml | Gibco | 10378016 |
| 0,5× N-2 supplement (stock: 100X) | 1,25 ml | Gibco | A1370701 |
| 0,1   mM cAMP (stock: 10mg/ml) | 922,5 μl | Sigma Aldrich | A6885 |
| 0,025% Bovine Serum Albumin Fraction V  (BSA) solution (stock: 7,5% solution) | 833,33 μl | Gibco | 15260037 |
| 55 μM 2-Mercaptoethanol (stock: 50mM) | 275 μl | Gibco | 31350010 |
| 0,2 mM Sodium-L-Ascorbate (stock: 50mg/ml) | 197,5 μl | Sigma Aldrich | A4034 |
| 5 ng/mL NT-3 (stock: 100 μg/ml) | 12,5 μl | Peprotech | 0450-03-10 |
| 5ng/mL BDNF (stock: 100 μg/ml) | 12,5 μl | Peprotech | 0450-02-10 |
| 5 ng/mL GDNF (stock: 100 μg/ml) | 12,5 μl | Peprotech | 0450-10-10 |

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**cAMP (Sigma Aldrich,** A6885**):** stock is 10mg/ml in water. (The location is in the corner fridge in iPSC lab at -20 oC). It is stable at -20oC up to 3months

**Sodium-L-Ascorbate (Sigma Aldrich, A4034):** stock is 50mg/ml in water. (The location is in the corner fridge in iPSC lab at -20 oC). It is stable at -20oC up to 6 months

**NT-3 (Peprotech, 450-03):** stock is 100 μg/ml in water. (The location is in the corner fridge in iPSC lab at -20 oC). It is stable at -20oC up to 6 months

**BDNF (Peprotech, 450-02):** stock is 100 μg/ml in water. (The location is in the corner fridge in iPSC lab at -20 oC). It is stable at -20oC up to 6 months

**GDNF (Peprotech, 450-10):** stock is 100 μg/ml in water. (The location is in the corner fridge in iPSC lab at -20 oC). It is stable at -20oC up to 6 months

The cell pellets were collected for further analysis (RNA isolation, cDNA synthesis and qPCR). Cell suspensions were centrifuged for 2 minutes at 1000RPM. Supernatant were removed, cell pellets were re-suspended with PBS. The process was repeated once. Cell pellets were put into dry ice for 2 minutes, afterwards put into –80oC.

The wells with coverslips were fixated for immunostaining. Medium was removed and cells were washed with PBS (1X). 4% paraformaldehyde (PFA) were added to wells and incubated for 10 minutes at room temperature. PFA was removed and wells were washed with PBS for 2-3 times. For long term storage, PBS-Azide was added.