SHSY5Y Differentiation Protocol

Equipment:

- Trypsin
- Phosphate buffered Saline
- 75 cm^2 culture flask
- All-trans retinoic acid (50 mM stock)
- B27 supplement (50x stock)
- Potassium chloride (1M stock)
- dibutyryl cyclic AMP (1M stock)
- Brain-derived neurotrophic factor [BDNF] (100x stock)
- Fetal bovine serum
- EMEM
- Neurobasal
- Basic Culture Medium
- Trypsin

Differentiation Medium 1 (50 ml)

- 48,5 ml EMEM
- 500µl Pen/Strep
- 1,3 ml FBS
- 100µl Retinoic acid (50mM stock)

Differentiation Medium 2 (50 ml)

- 49,5 ml EMEM
- 500 µl Pen/Strep
- 500 µl FBS
- 100µl Retinoic acid (50mM stock)

Differentiation Medium 2 (50 ml)

- 47 ml Neurobasal
- 500 µl Pen/Strep
- 1 ml KCl (1M stock)
- 1 ml B27 (50x stock)
- 500 µl Glutamax I
- 250 µl BDNF (100x stock)
- 100 µl db-cAMP (1M stock)
- 100µl Retinoic acid (50mM stock)

Retinoic acid is light sensitive, add immediately before use. Keep medium up to 2 weeks at 4°C.

Differentiation takes 18 days and is then 14 days stable, depending on cell passage.

Day 0 Plating Cells for Differentiation

- Rinse undifferentiated cells with 1x PBS, aspirate, and then trypsinize using 1-2 ml warmed 1x 0.05% Trypsin-EDTA
- -When cells are in trypsin, incubate for approximately 3 min in an incubator.
- Quench the trypsin by adding 10 ml Basic Culture Medium, rinse the sides of the flask or dish, and gently triturate 1-3 times. Transfer contents to a 15 ml conical tube.
- Centrifuge for 2 min at 1,000 x g and aspirate the media while being careful not to disturb the pellet.
- Resuspend pellet in 5 ml Basic Culture Media and triturate 1-3 times.
- Count cells using a hemocytometer, then dilute using Basic Growth Media to 50,000 cells/ml.
- Plate 2 ml of cells per 35 mm2 dish for a total of 100,000 cells per dish and place back into incubator

Day 1 Change Media (Differentiation Media 1)

- Aliquot 50 ml of Differentiation Media 1 and incubate in a 37 °C water bath.
- When media is warmed, allow it to equilibrate in an incubator (37 °C, 5% CO_2) for at least one hour to establish a proper pH balance prior to use.
- Add Retinoic Acid (RA) to warmed and equilibrated media immediately before adding media to dishes. Note: Retinoic acid is light sensitive and should be stored in dark bottles at 4 °C
- Gently aspirate off old media and discard.
- Add 2 ml Differentiation Media 1 with RA per 35 mm^2 dish and return to incubator

Day 3 Change Media (Differentiation Media 1)

- Repeat Day 1 protocol

Day 5 Change Media (Differentiation Media 1)

- Repeat Day 1 protocol

Day 7 Split Cells 1:1

- Add RA to warmed and equilibrated Differentiation Media 1 immediately before adding media to dishes.
- Gently aspirate off old media and discard.
- Add 200 µl warmed 0.05% 1x Trypsin EDTA per 35 mm^2 dish and warm in incubator approximately 2-3 min or until cells are visibly lifted fromthe plate as observed under a microscope.
- Quench the trypsin by adding 2 ml Differentiation Media 1 with RA per 35 mm^2 dish and use the media to rinse remaining neuronal cells off the plate. Then transfer contents to a 50 ml conical tube.
- Note: During trypsinization steps, do not trypsinize too many dishes at once. This helps to ensure neuronal cultures are not incubated in trypsin for too long, which can be cytotoxic
- Combine contents from up to 10 dishes in the 50 ml conical tube and gently triturate slowly up and down no more than five times with a 10 ml plastic pipet
- Aliquot 2 ml cell suspension into fresh 35 mm^2 dishes and return to incubator

Day 8 Change Media (Differentiation Media 2)

- Add RA to warmed and equilibrated media immediately before adding media to dishes.
- Gently aspirate off old media and discard.
- Slowly add 2 mL Differentiation Media 2 with RA per 35 mm^2 dish and return to incubator. Do not allow neurons to be exposed to air for an extended period of time as they can dry out quickly.

Day 9 Prepare Extracellular Matrix (ECM) Coated Dishes

- Thaw one vial of ECM solution on ice and dilute 1:100 into cold DMEM.
- Dispense 2 ml of mixture into each 35 mm2 dish and ensure the entire base of the dish is covered.
- Place in an incubator (37 °C, 5% CO2) for 1 hr or overnight.
- Aspirate mixture and allow to air dry for approximately 1 hr in a hood. Store at room temperature for up to 2 months.

Day 10 Transfer Cells onto ECM Coated Plates 1:1

- Add RA to warmed and equilibrated media immediately before adding media to dishes.
- Gently aspirate off media and discard.
- Add 200 µl warmed trypsin to each 35 mm2 dish and allow to incubate at room temperature for approximately 1-2 min or until neurons are visibly lifted from the dish as observed under a microscope.
- Note: Execute this trypsinization step at room temperature so as not to over-incubate neurons with trypsin and cause damage. Neurons release from plates much faster than epithelial-like cells at this stage.
- Quench the trypsin by adding 2 ml Differentiation Media 2 per 35 mm^2 dish and use the media to rinse remaining neuronal cells off the plate. Then transfer contents to a 50 ml conical tube.
- Combine contents from up to 10 dishes in the 50 ml conical tube and gently triturate slowly up and down no more than five times with a 10 mlplastic pipet.
- Dispense 2 ml cell suspension into ECM-coated 35 mm^2 dishes and return to incubator.

Day 11 Change Media (Differentiation Media 3)

- Add RA to warmed and equilibrated media immediately before adding media to dishes.
- Gently aspirate off old media and discard.
- Slowly add 2 ml Differentiation Media 3 with RA per 35 mm^2 dish and return to incubator. Do not allow neurons to be exposed to air for an extended period of time

Day 14 Change Media (Differentiation Media 3)

- Repeat Day 11 protocol

Day 17 Last Media Change (Differentiation Media 3)

- Repeat Day 11 protocol

Day 18 Neuronal Cultures Ready to Use

- Change media to fresh Differentiation Media #3 with RA every 3 days to maintain neuron health.

Note: Cells should be differentiated into neurons and exhibit a neuronal phenotype. Cultures are typically stable for up to 14 days following terminal differentiation, however duration of neuron viability is dependent on passage number of the undifferentiated cells at the start of differentiation. Higher passage numbers yield differentiated neurons with a shorter useful lifetime.