MIDBRAIN DOPAMINE NEURON PLATING AND CULTURING PROTOCOL

Coating Coverslips

Washing coverslips

1. boil round coverslips with ~~Ø~~12mm (fisher#12-545-81) in **1M HCl** for 5min
2. let cool down
3. wash 3x with ddH_2O
4. 30min 100% EtOH
5. wash 3x with ddH_2O
6. cover beaker with aluminum foil and autoclave for 30min

Coating coverslips

1. distribute coverslips with flamed forceps in 24 wells
2. 2h 37^oC **in 0.001% Poly-L-Ornithine** (Sigma P4957, 0.01%) **in ddH_2O** (500µl each well)
3. wash 2x with ddH_2O

Before beginning dissection, prepare Dissociation media as follows:

Dissociation media (DM)
15.5mL Cysteine Water*
400 units papain (Add papain to cysteine water and mix before adding other reagents)
4mL DISCO**
20µL 5N HCl
20µL kynurenic acid (0.5M stock)
40µL phenol red

***Cysteine Water**
44.1mL H_2O
7mg Cysteine (RT chemical shelf)
855µL 100mM $CaCl_2$

****DISCO**
Concentrations are final concentrations in 50mL
NaCl (116mM)
KCl (5.4mM)
$NaHCO_3$ (26mM)
NaH_2PO_4·H2O (2mM)
$MgSO_4$ (1mM)
EDTA (0.5mM)
Glucose (25mM)

Once media is prepared, sterile filter using a 60cc syringe/syringe filter into a flat bottom plastic tube, warm to approx. 37^{o}C for roughly 30 minutes to allow cysteine to activate papain. While this incubates prepare for dissection.

Substantia Nigra Dissection and Plating:

Pups should be 0-3 days of age.

- Place two 10 cm petri dishes on ice and fill with ice cold Gey's Balanced Salt Solution.
- Remove 4 pups from mother and keep them in a small box with bedding or paper towels. Loosen box lid in a plastic bag with isoflurane soaked guaze.
- Once pups stop moving, but before they turn purple, remove them from bag and remove the head with large, surgical scissors. Cut skin to expose skull and cut skull around skullcap with small surgical scissors. Using a spatula, gently scoop brain out of skull, starting by loosening brain at the front and drop into the Gey's solution on ice.
- Remove all brains and then begin dissection. Flip brain so that the ventral face is up. Use fine tip forceps to hold the brain down and gently push the fold of the brain back. Make a perpendicular cut behind and forward and flip the slice to take tissue below the aqueduct. Cut tissue into 2 or 3 small chunks. Transfer chunks to fresh Gey's in the other plate using a 25 ml pipet.
- In TC hood, transfer tissue chunks to DM, transferring as little liquid as possible. Add a tiny stir bar from alcohol. Make two holes in the cap of the tube with a 16-18 gauge needle. Insert needle down just into solution for bubbling with oxygen. Tissue and DM is gently stirred and oxygenated at 37^{o}C for 1-2 hours.
- While tissue is dissociating, prepare **Glial Medium***** and coat 12 mm Poly-L-Lysine coverslips (BD354085) with laminin. Place each coverslip in a 35 mm petri dish using sterile forceps. To coat coverslips, add 50 ul of laminin (1mg/ml stock for 5 ug/ml final concentration) to 10 ml cold MEM, then sterile filter and pipet 100 ul onto each coverslip. Allow coverslips to set at RT for about 1 hour.

Glial Medium (GM)***

65 ml MEM with no glutamine (Gibco #11090-081)
50 ml fetal bovine serum
10 ml Pen/Strep (5000 units/ml stock)
100 ul Insulin (25 mg/ml stock)
3.8 ml 45% Glucose (20.25 g in 45 ml dH_2O, must be warmed to go into solution)
1.23 ml 200 mM Glutamine (292.2 mg in 10 ml dH_2O, freeze in 1.25 ml aliquots)

- After incubation, bring tissue to TC hood. Allow tissue to settle to the tube bottom, and transfer to a 50 ml conical tube using a 10 ml pipet. Try to transfer as little medium and with as few air bubbles as possible.
- Add DNAse I solution to final concentration of 30 ug/ml for additional 3 minutes.

- Wash tissue 2x with ~25 ml GM, allowing tissue to settle to the bottom before removing supernatant. After the second wash, aspirate tissue and 2 ml of medium with a 10 ml pipet to a 15 ml conical tube for tritration.
- Tritrate tissue, starting with a 5 ml pipet and working down through 4 increasingly smaller tipped Pasteur pipets with cotton in the top (Pasteur pipets were prepared by firing in a Bunsen burner for decreasing tip diameters).
- Pass the triturated tissue through 70-um cell strainer. Wash the strainer with GM.
- The cell suspension is then centrifuged for 10 minutes at 1500 RPM at 4 ºC. Supernatant is decanted and 10 ml of GM is added and mixed to homogenize tissue pellet.
- Taking from the upper middle level of the preparation, pipet 100 uL of this suspension dropwise onto each coverslip. Coverslips are then incubated at 37^{o}C for 1 - 2 hours before being gently flooded with 2 ml of **Neuronal Media 10 (NM10)** and returned to the incubator. Feed cells every 7 days, by replacing 1 ml of medium with fresh NM+. Cells are ready for transfection and electrophysiological recording in about 10 days.

Neuronal Media 10 (NM10):
Gibco #11090-081 MEM w/no glutamine (250ml → filter)

-Add 100 ul insulin (25 mg/ml)
-Add 1 ml transferrin (100 mg/2 ml GBSS)
-Add 3.8 ml Glucose (45%)
-Mix in 15 ml tube with about 5 ml MEM and add to filter

-HI horse serum – 50 ml
-HI fetal calf serum (FCS) – 5 ml
-HAMS F12 – 195 ml (Gibco #11765-054 w/1 mM glutamine)

Then filter

Kynurenate Acid Stock (0.5M)
Add 2.6 ml of 1.0N NaOH to 250 mg bottle
Aliquot 50 ul/tube