Madeline Lancaster

Differentiation of hESCs to Cerebral Organoids

**Materials**

* DMEM/F12: Invitrogen cat#11330-032
* KOSR: Invitrogen cat# 10828-028
* GlutaMAX: Invitrogen cat#35050-038
* P/S: Penicillin/Streptomycin: Sigma cat#P0781
* MEM-NEAA: MEM-Non-essential amino acids: Sigma cat#M7145
* 2-Mercaptoethanol
* bFGF (FGF2): Peprotech cat#100-18B
	+ 10ug/ml solution prepared by reconstituting 50ug in 5ml PBS +0.1% BSA
	+ Aliquot and store at -20C for up to 1 year
* 0.05% Trypsin/EDTA solution: Invitrogen
* Trypsin inhibitor: Sigma cat# T6414-100ML
* Heparin: Sigma cat#H3149
	+ 5mg/ml solution in PBS – store at -20C for up to 1 year
* Dispase: Invitrogen cat#17105-0412q
* Rock inhibitor Y27632, 5mg: VWR cat# 688000-5
	+ Working solution prepared by reconstituting 5mg in 2.96 ml water
* N2 supplement: Invitrogen cat# 17502048
	+ Aliquot and store at -20C for up to 1 year
* B27-vit. A supplement: Invitrogen cat# 12587010
	+ Aliquot and store at -20C for up to 1 year
* B27+vit. A supplement : Invitrogen cat# 17504044
	+ Aliquot and store at -20C for up to 1 year
* Neurobasal medium: Invitrogen cat# 21103049
* Sterile D-PBS w/o Ca and Mg
* Insulin solution: Sigma cat# I9278-5ML
* Spinner flask, 125ml size: Corning cat# 4500-125

**Media**

Low bFGF hES media (500 ml) – Store at 4C up to 2 weeks

* 400 ml DMEM/F12
* 100 ml KOSR
* 15 ml ES-quality FBS
* 5 ml GlutaMAX
* 5 ml MEM-NEAA
* 3.5 ul 2-Mercaptoethanol
* Filtered using 0.22 um filter
* 4 ng/ml bFGF (Add just before use)

Dispase solution (10 ml) – Store at -20C up to 6 months

* 5 mg Dispase
* 5 ml DMEM/F-12
* Filtered using 0.22 um filter

Heparin solution (10 ml) – Store at -20C up to 6 months

* 2 ml of 5mg/ml solution Heparin in PBS
* 8 ml DMEM/F12

Neural induction media (50 ml) – Store at 4C for up to 2 weeks

* 50 ml DMEM/F12
* 0.5 ml N2 supplement
* 0.5 ml Glutamax supplement
* 0.5 ml MEM-NEAA
* 50 ul Heparin solution
* Filtered using 0.22 um filter

Differentiation media (250 ml) – Store at 4C for up to 2 weeks

* 125 ml DMEM/F12
* 125 ml Neurobasal
* 1.25 ml N2 supplement
* 2.5 ml B27 +/- vitamin A supplement
* 62.5 ul insulin
* 87.5 ul 2-ME solution (1:100 dilution in DMEM/F12)
* 2.5 ml Glutamax supplement
* 1.25 ml MEM-NEAA
* 2.5 ml P/S

Procedure

**Making embryoid bodies**

1. When hESCs colonies are ready for splitting, wash colonies with D-PBS w/o Ca and Mg and add 1 ml Dispase solution for each well of 6-well plate.
2. When the colony edges begin to curl off plate, remove dispase solution and wash with 1 ml D-PBS w/o Ca and Mg. Remove colonies by spraying with 1 ml hES media using a P1000 tip, 3 times (3 ml total) and transfer to a 15-ml conical tube being careful to limit the disruption of colonies.
3. Allow colony clumps to settle for 3 min and aspirate supernatant gently with a pipette.
4. Resuspend colonies in 1 ml Trypsin/EDTA and incubate 2 min at 37C. Add 1 ml trypsin inhibitor and triturate using a P1000 tip until solution becomes cloudy with single cells. Take 5ul for cell counting, then add 8 ml low bFGF hES media.
5. Centrifuge cells at 270xg for 5 min and in the meantime count live cells.
6. Resuspend in appropriate volume of low bFGF hES media +1:100 Rock inhibitor (9000 live cells/150ul).
7. Plate 150ul in each well of a low attachment 96-well plate.
8. Change the medium every other day for 6-7 days, with Rock inh. for the first 4 days. Leave out low bFGF after 4 days.

**Making primitive neuroepithelia**

1. When EBs are about 500-600um in diameter and begin to brighten and have smooth edges (day 6 or 7), transfer EBs to neural induction media in a low cell binding 24-well plate (1-2 per well) using a cut P200 tip to carefully transfer without disrupting.
2. Feed the EBs with neural induction media every other day. Aggregates should become brighter around the outside with visible neuroepithelia after a few days in the neural induction media (after 4-5 days); healthy cell aggregates should have smooth edges.

**Making cerebral tissue**

1. When neuroepithelia are evident, transfer the aggregates to Matrigel droplets.
	1. Using a cut P200 tip, transfer aggregates one by one to dimpled Parafilm (cover a tip holder with a sheet of parafilm and push parafilm into holes to create dimples).
	2. Remove excess media and add droplets of Matrigel to each aggregate. Position each aggregate in the center of the droplet using a pipette tip.
	3. Place parafilm sheet in a 6 cm dish in the 37 incubator for 30 min to allow matrigel to polymerize.
	4. Add Differentiation media –vitamin A and remove matrigel droplets from parafilm by agitating until they fall of the sheet.
	5. Feed every other day.
2. When tissues begin to show more complex neuroepithelia with some budding outgrowth and radial processes in the matrigel (after 3-4 days), transfer 6cm plate to a shaker (85 rpm) in Differentiation media +vitamin A, or transfer the matrigel droplets using a cut P1000 tip to a spinner flask (size 125ml) with 75ml Differentiation media +vitamin A spinning at 25rpm in a standard TC incubator.
3. Change media every 3-4 days if on the shaker or every week for the spinner flask and monitor for morphology.
4. Cerebral organoids are ready for analysis when tissue completely fills matrigel droplet and has been growing on shaker or in spinner flask for at least 2 weeks.