Madeline Lancaster

Cerebral organoids Version 2.1 from feeder-free cells grown in Stemflex

**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: 50 mM solution of 2ME: Life Technologies #31350-010
* 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
* Bovine Serum Albumin (BSA): solid or ready made 10% solution in sterile Water or PBS
* Heparin: Sigma cat#H3149
	+ 1mg/ml solution in PBS – store at -4C
* Accutase: A6964 SIGMA
* 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
* Vitamin C solution: Dissolve 352 mg Ascorbic Acid in 50 ml DMEM/F12, Store in dark at 4C
* hES quality FBS
* Low attachment 96-well plates: CLS7007 SIGMA
* Low attachment 24-well plates: CLS3473 SIGMA
* Regular Matrigel: Corning # 354234
* CHIR: Tocris #4423 - Prepare 3 mM stock (1000x) in DMSO
* Absorbable sutures: Ethicon Vicryl, violet colored, polyglactin, 5-0 size

**Media**

Low bFGF hES media (50 ml) – Store at 4C up to 1 month

* 40 ml DMEM/F12
* 10 ml KOSR
* 1.5 ml ES-quality FBS
* 0.5 ml GlutaMAX
* 0.5 ml MEM-NEAA
* 100 ul of 50mM 2-ME
* Filtered using 0.22 um filter
* 4 ng/ml bFGF (Add just before use) (1:2500)
* 1:100 Rock inhibitor (Add just before use)

Neural induction media (100 ml) – Store at 4C for up to 1 month

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

Improved Differentiation Media -A (250 ml) – Store at 4C for up to 1 month

* 125 ml DMEM/F12
* 125 ml Neurobasal
* 1.25 ml N2 supplement
* 5 ml B27- vitamin A supplement
* 62.5 ul insulin
* 250ul of 50 mM 2-ME solution
* 2.5 ml Glutamax supplement
* 1.25 ml MEM-NEAA
* 2.5 ml P/S

Improved Differentiation Media +A +1.0 mg/ml NaHCO3 (500 ml) – Store at 4C for up to 1 month

* 250 ml DMEM/F12
* 250 ml Neurobasal
* 2.5 ml N2 supplement
* 10 ml B27+ vitamin A supplement
* 125 ul insulin
* 500 ul of 50mM 2-ME solution
* 5 ml Glutamax supplement
* 2.5 ml MEM-NEAA
* 5 ml P/S
* 500 mg NaHCO3
* 5 ml Vitamin C solution (40mM stock)

Improved Differentiation Media +A +1.5 mg/ml NaHCO3 (500 ml) – Store at 4C for up to 1 month

* 250 ml DMEM/F12
* 250 ml Neurobasal
* 2.5 ml N2 supplement
* 10 ml B27+ vitamin A supplement
* 125 ul insulin
* 500 ul of 50mM 2-ME solution
* 5 ml Glutamax supplement
* 2.5 ml MEM-NEAA
* 5 ml P/S
* 750 mg NaHCO3
* 5 ml Vitamin C solution (40mM stock)

Improved Differentiation Media + MG (50 ml) – Make fresh

* 50 ml Improved Differentiation Media +A+1.5– Keep cold!
* 1 ml Matrigel – Slowly thaw on ice and add slowly to cold media to dissolve

Procedure

**Preparation of fibrous microscaffold**

1. Remove suture from protective wrapping and starting from the free end of the suture, begin shaving with an angled scalpel blade. Shave on a sterile surface, such as an autoclaved steel instrument tray. Angle the sterile blade and shave away from your body to obtain microfibers of approximately 1 mm in length. Shave a total of 5 cm suture length.
2. Pipette 5 ml hES media without bFGF onto the fibers. Then cut a P1000 tip and pipette fibres up and down to fully immerse.
3. Transfer media with fibers to a 15 ml conical. Add 10 ml hES media without bFGF to bring it to a total of 15 ml. This is a 2X fiber solution.

**Making embryoid bodies**

1. When hESCs colonies are ready for splitting, wash colonies twice with 600 ul EDTA solution, then leave 600 ul EDTA on for 4 min.
2. Aspirate EDTA and use mTESR to spray off quantity needed for splitting (ie. 1/3 or 1/6 of the well). Suck off any remaining mTESR.
3. Add 500ul Accutase and incubate at 37C for another 4 min.
4. Tap the plate vigorously to remove cells and add 500 ul mTesr and pipette up and down until single cell suspension (approximately 4-5 times). Transfer all 1 ml to a 15-ml conical tube and take 5 ul for counting to mix with 5 ul Trypan blue. Repeat this in order to get two replicate counts.
5. Spin cells at 200xg for 4 min. During spin, count live cells from the two replicates and take the average.
6. Remove supernatant and resuspend in 1 ml Low bFGF hES media + 1:100 Rock inhibitor.
7. Prepare a tube with 75ul Low bFGF hES media per well and add an equal volume of 2X fibers in Low bFGF hES media (FGF and Rock inhibitor will need to be added to the volume of fibers). Be careful when pipetting fibers to use a cut P1000 tip and agitate between pipetting to be sure to pipette the fibers into the tip.
8. Transfer appropriate volume of cells to media + fibers in order to plate 18000 cells per well.
9. Using a cut P200 tip, transfer 150 ul of fiber media + cells to each well of a low adhesion 96 well U-bottom plate. Again, be careful when pipetting fibers to agitate between pipetting to be sure to pipette the fibers into the tip.
10. Change medium on day 3 with hES media without bFGF or RI.

**Making primitive neuroepithelia**

1. When EBs are about 500-600um in thickness and begin to brighten and have smooth edges (day 5 or 6), transfer EBs to neural induction media in a low cell binding 24-well plate (1 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 20 min to allow matrigel to polymerize.
	4. Add Neural Induction and remove matrigel droplets from parafilm by agitating until they fall of the sheet.
2. 2 days after Matrigel embedding (usually day 13) change media to Improved Differentiation Media -A + 3uM CHIR. After three days of CHIR, change back to Improved Differentiation Media –A without CHIR.
3. 2 days after finishing CHIR treatment, feed organoids and transfer to the orbital shaker.
4. 2 days after transfer to shaker, feed organoids with Improved Differentiation +A +1.0mg/ml NaHCO3. Continue feeding organoids every 3-4 days.
5. At day 40 switch media to Improved Differentiation Media +MG.

Overview of timeline: