Madeline Lancaster

Generation of cerebral organoids using the SCT kit

**Materials**

* STEMdiff Cerebral Organoid kit: cat# 08570
* DMEM/F12: Invitrogen cat#11330-032
* 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
* 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**

Improved Differentiation Media -A (IDM-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 +1.5 mg/ml NaHCO3 (IDM+A) (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)

IDM+A + 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

**Making embryoid bodies**

1. When hESCs colonies (grown in Stemflex and on MG coated dishes) 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 add 500ul Accutase and incubate at 37C for another 4 min.
3. Spray the cells off with 500 ul Stemflex and pipette up and down until a 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.
4. Spin cells at 270xg for 4 min. During spin, count live cells from the two replicates and take the average.
5. Remove supernatant and resuspend cell pellet in 1 ml kit EB media + 1:100 Rock inhibitor (RI).
6. Prepare a tube with 150ul kit EB media + 1:100 RI per well planned, and transfer the appropriate volume of cells to the media to obtain 2000 cells per well planned. For example, if planning to generate 32 organoids (4 columns of a 96 well plate), prepare 4.5 ml EB media+RI and add 64,000 cells to it.
7. Transfer 150 ul of media + cells to each well of a low adhesion 96 well U-bottom plate.
8. Change approximately ½ to ¾ of the medium on day 3 (careful not to aspirate the EB) with kit EB media without RI.

**Making neuroepithelial buds**

1. On day 5 EBs should be large and bright and should have smooth edges. Change media to 150 ul of kit neural induction (NI) media.
2. On day 7, transfer the EBs 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 dish in the 37 incubator for 20 min to allow matrigel to polymerize.
	4. Spray the droplets off the parafilm sheet with 1 ml kit Expansion media into a 6-well dish. 16 droplets per well in 2 ml of media is sufficient.

**Making cerebral tissue**

1. 3 days after Matrigel embedding (day 10) change media to Improved Differentiation Media -A (IDM-A).
2. On day 13, remove the Matrigel either by carefully dissecting it off using ultrafine scalpels, or simply by pipetting up and down once through a 10ml pasteur pipette. The former will remove more of the Matrigel, while the latter is easier but will leave some Matrigel attached which can influence tissue structure. At this stage, move the organoids to a 6cm dish with 5ml IDM-A and move them to the shaker, shaking at 57rpm.
3. From day 17, organoids can be fed with IDM+A or kit Maturation media.
4. At day 30 being adding dissolved Matrigel 1:50 to the medium.

Overview of timeline: