Protocols for Embryonic Stem (ES) cell culture and Primary Neural Stem Cell Culture

Yingwei Mao, 8/12/04

ES protocol

#1 ES cell culture

Material:

1. ES base growth media 500 ml (SLE -)
   - 500 ml of DMEM (Invitrogen, 11965-092)
   - 50 ml of 10%FCS(from Transgenic core)
   - 27 ml of ES supplement
     - 5.4 mM HEPES final concentration
     - 27 μM β-ME
   - 5 ml of 100X Pen/Strep with glutamine (replenish every 2 weeks with fresh glutamine)

   Filter sterilze

2. ES growth media (SLE +)

   For every 50 ml of (SLE -)

   - add 50 μl of recombinant mouse LIF (Chemicon), final concentration 5 ng/ml (1000 units/ml)

3. ES supplement

   11.91 g HEPES (Sigma H4034, FW 238.3) – 0.1M
   35 μl β-ME (stock is 14.3M) – 0.5 mM
   500 ml DMEM
   Combine, filter and store as 27 ml aliquots in –80 deg freezer

4. LIF

   Chemicon, catalog # LIF2005
   Stock concentration 10 μg/ml
   Specific activity $\geq 1x10^8$ units/mg

0.25% Trypsin-EDTA (Invitrogen 25200-56)

All plates and dishes except for Petri dishes were coated with 0.1% gelatin (autoclaved, 30min at RT)

Method:

1. Aspirate media form ES cells
2. Rinse once with warm PBS and then remove PBS
3. Add 1ml trypsin (60mm dish) for 2-5 min
4. Add 1ml ES media to stop the trypsinization. Using a pipet to triturate the ES cells to single cell and transfer to a 15 ml tube.
5. Spin 5 min at 1600rpm
6. Aspirate supernatant and resuspend cell pellet in 10 ml of media
7. Count cells and seed $2X10^6$/60 mm dish or $8X10^5$/ 35mm dish, seed in high density, otherwise they differentiate (sign for differentiation: the edge of cells is not smooth)

8. Change media every other day and split once they become confluent.

#2 Freeze/ Thaw of ES cells
Freezing:
Material: fresh ES FCS+10% DMSO
Method:
1. Trypsinize and count cells
2. Spin down cells for 5 min at 1600rpm
3. Aspirate supernatant and resuspend in desired cell density (4-5X10^6/ ml) in freezing media
4. Aliquot 1ml of cells in freezing tube and place -80˚C O/N, next morning transfer to liquid nitrogen tank

Thawing:
1. Place the vial in a 37˚C water bath and shake continuously
2. Once media turned to liquid and dip into 70% ethanol
3. Transfer to a 15 ml tube containing 3 ml of ES media
4. Quickly spin down the cells and aspirate off the supernatant and resuspend in 5 ml ES media
5. Seed in 60 mm dish and the next day check the confluence of viable cells

#3 4-/4+ RA Differentiation protocol
Material: ES differentiation media (ES media without LIF and β-mercaptoehtanol)
Method:
1. Trypsinize ES cells and count cells
2. Resuspend in 8 ml of ES differentiation media at 1X10^6/ 10 cm Petri dish for 4 days without RA. After 48 h, change to the fresh differentiation media- transfer the embryoid bodies into a 15 ml tube and let them precipitate for 10 min, then remove the old media and resuspend in fresh 8 ml of media
3. After 96 h, change to fresh differentiation media with 1 uM RA for another 4 days using precipitation method; change media once in the middle of 4 days
4. At the end, transfer the embryonic bodies into a 15 ml tube and spin down at 1600 rpm for 5 min
5. Remove old media and wash once with 5 ml PBS, resuspend in 1 ml of trypsin , pipet up and down to dissociate into single cells
6. Add 3 ml differentiation media to the tube and count cells
7. Seed 2X10^5/ well in 12 well plate with 1 ml of differentiation media, change media every other day
8. After 7-12 days, fix and stain with Tuj-1 Ab or Hoechst dye

Neural stem cell protocol

#1 Isolation and growth
Material: Opti-MEM
Scissors, fine forceps, razor blade
Dissociation buffer
Trypsin inhibitor solution
Serum-free media (SFM)
Method:
1. Sacrifice P14 or P15 mice by CO_2 asphyxiation
2. Spray the mouse body with 70 % ethanol and cut the head off using scissors , then open skull using fine forceps and dissect the brain out
3. Place brain in 3 ml of cold Opt-MEM in 60 mm dish. With the under side facing up and using a clean razor blade, make coronal sections - first cut off olfactory

bulb, then make a second cut half way between the olfactory bulb and the hypothalamus. Finally, make a cut in front of the hypothalamus. Dissect out the SVZ region

4. Transfer the SVZ section to a 60 mm dish containing 2-3ml of ice-cold Opti-MEM
5. Under the a dissecting microscope, dissect out the lateral SVZ with fine forceps and place the pieces in another 60 mm dish containing 2ml cold Opti-MEM
6. Mince tissue with fine forceps
7. Transfer tissues to a 1.5 ml tube. Spin down at 500 rpm for 1 min at 4 ˚C
8. Remove media and transfer tissue into a 15ml tube containing 3 ml of dissociation buffer. 37 ˚C for 20 min and using 1 ml tip to pipette up and down for 10 times to dissociate tissue gently
9. Add 7 ml of trypsin inhibitor solution into tube and spin at 700rpm for 5 min at 10˚C
10. Remove supernatant and resuspend in 1ml SFM with growth factors. Dissociate by using 1ml pipette gently pipette up and down for 30 times
11. Filter through a 70 micron mesh into a 60 mm dish and rinse with 400 ul SFM
12. Count cells and seed 4 $X10^4$/60 mm dish (1900 cells/cm^2) in 3 ml of SFM with growth factors (from 5 P14 mice, normally can get 4-8$X10^5$ cells)
13. Change media every 3 days. After 2 days, neurospheres should be observed. If there are too much tissue debris, after 24 h , transfer NSC into a 15 ml tube and spin at 700 rpm for 3-5 min, remove old media and change to fresh media and new dishes
14. Passage at day 7.
    Two ways to dissociate neurospheres:
    1) Transfer them to a 15 ml tube and spin at 700 rpm for 5 min.-> Remove supernatant and leave 200 ul media. ->Using a p200 pipetman set to volume 200 ul, rinse the tip with medium, to avoid cell sticking inside the tip. Gently triturate for 40-50 times. Slightly tilt the pipetman and press tip against the bottom of the tube to generate a fair amount of resistance.-> count cells
    2) After spinning down neurospheres, remove old media and resuspend neurospheres in 0.5 ml of 0.25% trypsin-EDTA at 37˚C for 5 mins.-> Add 0.5 ml trypsin inhibitor solution and pipet up and down for 10-20 times using fire polished Pasteur pipette to dissociate them into single cell.-> Spin down and aspirate the supernatant. Resuspend cells in NSC media and count cells. (I found that trypsinization gives better separation and less cell damage).
15. Seed cells at 4.8x10^3/cm^2 to generate secondary neurospheres. Need 5-10 days and will give 3-5 fold more neurospheres.

Neurosphere maintenance (AWL notes)

1. Primary neurospheres - first ones to form after tissue dissociation and plating in vitro. Will take 7 days to form.
   Fresh growth factors are added every other day while all of the media is changed once every 3 days. To change media, collect all neurospheres, spin down as before and resuspend in fresh media without dissociation.
   After plating 4-8x10^5 cells from dissected SVZ, we can a total of 3x10^6 cells at the end of 7 days.
2. P1 neurospheres - dissociate using a combination of trypsinization (3-5 min at room temp, followed by neutralization and trituration with a P1000 pipetman, 15-20 times). Spin out and plate 1 x 10^5 cells/60 mm dish (4800 cells/cm^2) in fresh media. After 6-7 days, we get 1x10^6 cells per dish.

3. We have been able to generate P4 neurospheres in this manner.

#2 NSC Differentiation

Method:

1. Coat dishes with poly-D-lysine and fibronectin: First coat dishes with 20 ug/ml poly-D-lysine in PBS for 1hour at RT and wash twice with PBS. Remove PBS and let it dry. Then coat with 1 ug/ml of fibronectin in PBS for 2-6 hours at 37 ˚C. Wash once with PBS before plating. Use 0.3 ml/well for 48 well plate, 0.5 ml/well for 24-well plate, 1ml/well for 12-well plate.
2. After dissociation, seed $4X10^4$/ well in 24-well plates ($2.1x10^4/cm^2$), $1X10^4$/well in 48 well plates ($1x10^4/cm^2$). Use NSC media with 0.5% FBS without growth factor. It was reported that addition of 1 uM RA or 20 ng/ml PDGF can increase the neuron number. To prevent massive cell death, NSC can be plated in presence of low bFGF(10ng/ml). But bFGF will increase astrocyte and reduce neuronal percentage.
3. Change every 3 days and after 7 days, immunostain the cells with different antibodies. Normally, neurons appear earlier. But major cell type is astrocyte.

Solutions:

100 ml SFM:
96 ml DMEM/F12 (Invitrogen, with Glutamine, without HEPES),
1 ml Penn/Strep,
1 ml N-2 supplement (to be aliquoted into single-use tubes and stored at -20 deg)
2 ml B-27 supplement (to be aliquoted into single-use tubes and stored at -20 deg)
50 uM β-mercaptoethanol,
20ng/ml EGF,
20ng/ml FGF2,
5 ug/ml Heparin

NSC medium
SFM without EGF, FGF and heparin

Dissociation buffer:
13.3 mg trypsin
6.67 mg Hyaluronidase
1.3mg Kynurenic acid
Add Kynurenic acid into 10 ml HiLo ACSF and incubate in 37˚C water bath for 20 min to dissolve the kynurenic, then add trypsin and hyaluronidase.

Trypsin inhibitor solution:
22mg trypsin inhibitor
1 ml DNase I (1 mg/ml stock)
Dissolve in 100ml DMEM/F12

125 ml HiLo ACSF:

7.75ml	2M NaCl
0.625 ml	1M KCl
0.4 ml	1M MgCl2
21.125ml	155mM NaHCO3
1.25ml	1M glucose
0.1157ml	108mM CaCl2
2 ml	Penn/strep
93.73ml	H2O

Cell culture vessel surface areas

60 mm	21 cm^2
35 mm	8 cm^2
6 well	9.5 cm^2
12 well	3.8 cm^2
24 well	1.9 cm^2
48 well	0.95 cm^2