

# **Brain Cell Cultures 625**

## **Lab Manual**

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## **I. Routine Procedures in the hood**

- Turn on the UV light for at least 15 min before use.
- Supplies defined for cultures should always stay in the hood (pen, forceps).
- Clean working surface of the hood with alcohol **before and after** your usage.
- Always clean the aspiration line with bleach (diluted) after usage.
- Always empty the liquid waste in the small sink in the chemical hood **after your usage**.
- Follow **sterile procedures** when you are working in the hood.

**WE NEED EVERYONE'S COOPERATION TO KEEP AN EFFECTIVE WORKING ENVIRONMENT!**

## II. SUBCULTURING C6 GLIOMA CELLS

1. Aspirate medium from culture
2. Rinse cells with 5 ml of PBS buffer (pre-warmed to 37°C).
3. Aspirate PBS buffer
4. Add 1 ml trypsin (2.5mg/ml, pre-warmed to 37°C) into the flask (or a petridish), let it cover the entire monolayer
5. View cells under the microscope, aspirate the trypsin solution when cellular processes are retracted and cells are separated from each other (do not wait till the monolayer is detaching)
6. Add 10 ml of complete medium into the flask (or a petridish), make a cell suspension by trituration
7. Count cells (see next page)
8. To keep one stock flask (or a petridish), add 0.5ml suspension into a new flask (or a petridish) and add 14 ml of a complete medium (10 ml for the petridish).
9. Transfer 50  $\mu$ l, 100  $\mu$ l of cell suspension ( $\sim 1 \times 10^4$ ) into each chamber (of the chamber slide) and add 1 ml of the complete medium.
10. Refeed cells on the following Monday.

- C6 rat glioma cell: glial cell line.
- Complete medium:

880 ml of Han's F-12 (C6)

100ml fetal bovine serum

10 ml L-glutamine

10 ml penicillin/streptomycin

### III. QUANTIFICATION OF CELLS

#### PROCEDURE

In a microcentrifuge tube take 15  $\mu$ l cell suspension +15  $\mu$ l 0.4% trypan blue.  
Mix gently and fill the counting chamber (< 20 $\mu$ l) with the help of a pipette.  
Count all unstained (viable) and stained (dead) cells in 4 large corner squares in the counting chamber. Count cells that touch the left and upper lines and disregard those touching the right and lower lines.  
Dilution factor = 2

Total cell number:

$$\text{Cells/mL} = \frac{\text{Number of cells in 4 squares} \times 10 \times 1000 \times 2}{4}$$

Frequency of viable cells:

$$\% \text{ of viable cells} = \frac{\text{viable (unstained) cells counted}}{\text{total cells counted}} \times 100$$

Remove the cover glass and both coverglass and hemacytometer into a beaker of dH<sub>2</sub>O.  
Clean and dry the hemacytometer and coverglass.

## IV. IMMUNOFLUORESCENCE OF NEURONAL CELLS

### PROCEDURE

1. Remove medium from cultured cells and wash 2X (2 times) with 1 ml PBS. (add PBS against the wall, slowly)
2. Fix cells with 4% paraformaldehyde (in PBS) for 20 min at room temperature (RT).
3. Rinse cells 4x with PBS (1 ml).
4. Block cells in a Blocking buffer (10% normal goat serum + 0.4% Triton X-100 + 0.5% BSA in PBS) for 30min at RT.
5. Remove the blocking buffer and incubate cells in **1st Ab** diluted with Blocking buffer for 1 hr RT (400 $\mu$ l/well is sufficient for 4-chambered slides).
6. Wash 4X with PBS.
7. Add **2nd Ab** diluted with blocking serum and incubate for 1 hr at RT. (Keep it in dark, wrapped with foil).
8. Rinse 4X with PBS.
9. Aspirate PBS. Remove the plastic compartment (clear the mounting residuals with a blade). Keep the monolayer moistured with PBS.
10. Mount slide with fluorescent mounting medium. Add one drop of mounting medium on top of slide and gently place a clean coverslip over it. Make sure there are no air bubbles trapped. Press lightly and wipe the extra mounting medium from the edges carefully. Let it dry and observe under the microscope. (Keep slides in dark at 4°C for storage).

**For astrocytes, 1<sup>st</sup> Ab (primary)-rabbit anti GFAP (glial fibrillary acidic protein) 1:50-100. 2<sup>nd</sup> Ab (secondary)-goat-anti rabbit IgG-Alexa Fluor 594 conjugated, 1:200.**

**For neurons, 1<sup>st</sup> Ab (primary)-mouse anti-tubulin (III), 1: 50-100. 2<sup>nd</sup> Ab (secondary)-goat-anti mouse IgG-Alexa Fluor 488 conjugated, 1:200.**

## V. PRIMARY CORTICAL NEURONAL CULTURE

### PROCEDURE

#### A. Dissection of prenatal brain

1. Anesthetize E17 pregnant rat with ether and Spray the rats with 75% ethanol. Remove the uterus along with the embryos and put it in a petridish containing ice-cold HBSS.
2. Take the petridish to the hood. Perform rest of the steps in sterile conditions.
3. Isolate each embryo from the surrounding amnio-chorionic membranes with scissors. Hold the embryo securely around the neck with forceps and pin it on paraffin with the help of sterile needles. Wipe off any blood with a cotton tip applicator.
4. Open the skull with iridectomy scissors.
5. Remove the meninges and take out the cortices. Put cortices in ice-cold  $\text{Ca}^{++}/\text{Mg}^{++}$  free HBSS (5-10 ml). Remove cortices from all the fetuses and pool them in 10 ml HBSS.
6. Wash the cortices with HBSS.

#### B. Preparation of neuronal enriched cultures.

1. After dissecting the cortical tissues, mince it into small pieces with a scissors in a petridish containing ice-cold HBSS. Transfer the tissue in a 50 ml sterile tube. Spin it for 2 min. at room temperature (200-500g) and aspirate the supernatant.
2. Resuspend tissue in 10 ml HBSS (2 ml stock + 8 ml HBSS) containing 0.5 mg/ml trypsin. Incubate at 37°C for 25 min. in shaker bath.
3. Spin briefly (2 min., 200g), aspirate trypsin solution with a pipette and rinse cells 3 times with 3 ml HBSS.
4. Add 4ml of complete EMEM (CEMEM), triturate 70x with a 10-ml pipette.
5. Filter the cell suspension through a cell strainer fitted with 70  $\mu\text{m}$  mesh in to a 50-ml sterile tube. Add another 3 ml CEMEM to rinse the mesh.
6. Take 15  $\mu\text{l}$  of filtrate and add 15  $\mu\text{l}$  trypan blue to check the viability of the cells. Live cells should not take up the dye. Count cells in a hemacytometer and calculate the concentration (dilution factor =2).
7. Dilute cell suspension to  $2 \times 10^6$  dissociated cell/ml and **plate 4-chamber slides by adding 0.5 ml in each chamber** and 0.5ml per well ( $1 \times 10^6$  cells) in 24-well plates. Add 2.5ml suspension in each well of 6-well plate/petridish ( $5 \times 10^6$  cells).
8. Incubate cells at 37°C with 5%  $\text{CO}_2$ +95% air.
9. Change with fresh complete medium after 45 min.
10. Treat cells with CEMEM containing 8  $\mu\text{M}$  ara-c on day 4 for 3 days (DIV 4, 5 & 6).
11. Refeed cells with drug-free CEMEM on day 7.
12. Grow cells for another 4 days and do experiments.

### PREPARATION

- #### A. Poly-D-lysine (PDL) treatment - One day before the experiment (in the hood).

1. Use 4-chamber slides for Immunohistochemistry, 6-well plates or 35mm petridishes for western blot analysis and 24- well plates for flux assay.
2. Add 1-4ml of PDL (0.2mg/ml in ddH<sub>2</sub>O) into each well/plate.
3. Leave them in the CO<sub>2</sub> incubator overnight.
4. Aspirate PDL next day and rinse the plates with EMEM (2ml/well).
5. Transfer the plates back to the incubator.
6. PDL solution should be prepared fresh from a stock solution.
7. Immunohistochemistry can be done on coverslips in case chambered slides are not available.

#### Cleaning and autoclaving coverslips

1. Clean each coverslip with detergent. Wash and rinse well with ddH<sub>2</sub>O.
  2. Dry each coverslip with Kimwipe and store. Autoclave 30 coverslips in a petridish
  3. Place a coverslip in each well of sterile 6-well plates. Coat with PDL (as above).
- B. Cleaning / Autoclaving glassware and instruments (1 or 2 day before the expt).

#### 1. Instruments for dissection

Large scissors, forceps and scalpel - 2 sets. One steel box.

Small scissors, forceps and scalpel - 3 sets. One steel box.

#### 2. Petridish - 8, 100ml beakers - 4. Coverslips, if using

#### 3. Cell strainer and 200mesh(76µm)

#### 4. Cotton tip applicators

#### 5. Tips - P-1000, P-200, P-20

Petridish with paraffin - 4- Melt paraffin in each petridish over the hot plate (heating set 1). When cool, leave petridish open under UV overnight for sterilization. Next day wrap them in foil in the hood and store.

Wash steel boxes, instruments and glassware thoroughly with detergent. Wash and rinse with ddH<sub>2</sub>O. Wipe them with 70% alcohol. Wrap each set of instruments in a towel. Put large instruments in one box and small ones in the other. Put the boxes in sterilization pouch and seal. Put a little autoclave tape on each bag.

Assemble cell strainer and put it in a sterilization pouch and seal.

Put extra meshes in a sterilization pouch and seal.

Alternatively, 70µM sterile nylon cell strainers can be used (Fisher)

Wrap beakers and petridishes individually in foil. Put a little autoclave tape.

#### C. Preparation on the day of experiment.

Turn on the shaker bath and tissue culture water bath at 37°C. Keep CEMEM and trypsin in the bath. Put HBSS at 4°C.

#### D. Sterilization of laminar flow and equipment (1/2 to 1 hr before the Expt).

Wipe the interior surface of hood with 70% alcohol thoroughly. Wipe dissection microscope (if using) and pipette-aid with alcohol and put them in the hood. Put the autoclaved instrument boxes, beakers, 50ml tubes and cell-strainer in the hood. Turn on the UV light for 30 min.

## SOLUTIONS AND DRUGS FOR PRIMARY NEURONAL CELL CULTURE

Poly-D-Lysine - 0.2mg/ml ddH<sub>2</sub>O

PBS, sterile

Complete Eagle's modified essential medium (CEMEM)

Hanks balanced salt solution (HBSS) Ca<sup>++</sup>/Mg<sup>++</sup> free

Trypsin - 0.5mg/ml in HBSS

Trypan blue

0.02 M stock solution of Arabinofuranoside – 4.8mg/ml

CEMEM containing the final concentration of  $4-8 \times 10^{-6} \text{M}$

Poly-D-Lysine Stock Solution(50mg/ml)

Dissolve 500mg Poly-d-lysine in 10ml of ddH<sub>2</sub>O. Aliquot into 1ml. **Store at -20°C.**

Poly-D-Lysine Working Solution(0.2mg/ml)

Add 1ml aliquot of 50mg/ml PDL in 250ml ddH<sub>2</sub>O for a concentration of 0.2mg/ml. Mix well. Sterilize by filtration under the hood using 0.2µm filter.

**This solution should be made fresh each time.**

PBS (sterile)

30ml 1M K<sub>2</sub>HPO<sub>4</sub> + 15ml 1M KH<sub>2</sub>PO<sub>4</sub> ... pH is ~ 7.15

Adjust pH to 7.4 with 10N NaOH (100-150 µl)

Take 40 ml. Add 17g NaCl ... Make up the volume to 2 liter after adjusting pH to 7.4 with NaOH.

Filters sterilize under the hood.

Trypsin

2.5 g/Lit

i.e. 2.5 mg/ml

2.5mg ----- 1ml

0.5 mg -----  $\frac{0.5}{2.5}$  = 0.2 ml

For every 0.2ml trypsin, add 0.8 ml HBSS.

Or

Take 10 ml trypsin and add 40 ml HBSS - 0.5mg /ml

Complete Eagle's modified essential medium (CEMEM)

780 ml medium

100ml fetal bovine serum

100ml Horse serum

10 ml L-Glutamine

10 ml penicillin/ streptomycin (10,000 ug/ml)

Remove 220-ml medium from 1000ml flask. Add rest of the solutions. Store at 4°C.

Cytosine 1-b-D arabinofuranoside ( $2 \times 10^{-2} \text{M}$ )

Course 625  
Spring, 2004  
Instructor: Dandan Sun  
FW 243.2

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$$\begin{array}{l} 243.2\text{g/L} \text{ -----} 1\text{M} \\ 0.2432\text{g/ml} \text{ -----} 1\text{M} \\ 1\text{M} \text{ -----} 0.2432\text{g/ml} \\ \mathbf{2 \times 10^{-2} \text{ M}} \text{ -----} \frac{0.2432 \times 2}{100} = 0.004864\text{g/ml} \text{ -----} \text{Stock Soln} \end{array}$$

To make  $4 \times 10^{-6}\text{M}$  1X concentration  
20 $\mu\text{l}$  stock Soln. ----- Make up the vol. to 100 ml with Complete EMEM

## VI. PRIMARY CORTICAL ASTROCYTE CULTURE

### PROCEDURE

#### A. Dissection of neonatal brain

1. Anesthetize pups with ether in a dessicator in a chemical fume hood.
2. Remove the animals from the hood and spray alcohol over the animal. Decapitate the animal with a large scissors. Open the skull with iridectomy scissors. Remove the meninges and take out the cortices.
3. Put cortices in ice-cold  $\text{Ca}^{++}/\text{Mg}^{++}$  free HBSS (5-10ml) in a petridish. Pool tissues from two pups. Wash the cortices with HBSS 1x (one time).
4. Take the petridish to the laminar flow hood. Perform rest of the steps in sterile conditions.

#### B. Preparation of astrocyte enriched cultures.

1. Mince cortices into small pieces with a scissors in a petridish containing ice-cold HBSS (5ml). Transfer the tissue in a 50ml sterile tube. Spin it for 2 min. at room temperature (200-500g or 2500rpm) and aspirate the supernatant.
2. Resuspend tissue in 10 ml HBSS (2 ml stock + 8 ml HBSS) containing 0.5mg/ml trypsin. Incubate at 37°C for 25 min. in shaker bath.
3. Spin briefly (2 min., 200g), aspirate trypsin solution with a pipette and rinse cells 3 times with 3ml HBSS.
4. Add 3ml of complete EMEM (CEMEM), triturate 60x with a 10-ml pipette.
5. Filter the cell suspension through a cell strainer fitted with 70  $\mu\text{m}$  mesh (200 mesh) in a 50 ml sterile tube. Add another 3 ml CEMEM to rinse the mesh (total 6 ml suspension).
6. Take 15  $\mu\text{l}$  of filtrate and add 15  $\mu\text{l}$  Trypan blue to check the viability of the cells. Live cells should not take up the dye. Count cells in a hemacytometer and calculate the concentration (dilution factor =2).
7. Dilute cell suspension to  **$5 \times 10^4$  dissociated cell/ml and plate 4-chamber slides by adding 1ml in each chamber** and 1ml per well ( $5 \times 10^4$  cells) in 24-well plates. Add 4ml suspension per petridish or per well in 6-well plates ( $3 \times 10^4 / \text{cm}^2$ ).
8. Incubate cells at 37°C with 5%  $\text{CO}_2$ +95% air.
9. Change CEMEM next day and then after every 3 days.
10. Grow cells for 1-2 weeks. Refeed cells with medium containing 0.25-mM dibutyryl cAMP when they are confluent.
11. Experiments can be performed at DIV 12-30.

## PREPARATION

A. Collagen treatment - Can be done in advance.

\* Cleaning and autoclaving coverslips

1. Clean each coverslip with detergent.
2. Wash and rinse well with ddH<sub>2</sub>O.
3. Dry each coverslip with Kimwipe and store.
4. Autoclave 30 coverslips in a petridish.
5. Coat with collagen (as below).

\* Coating of 35mm sterile plastic petridish and coverslips with collagen (in the hood)

Coverslips need to be cleaned and autoclaved before coating.

1. Place a coverslip in each well of sterile 6-well plates.
2. Add 2ml of collagen (50µg/ml) into each dish (35mm)/plate.
3. Leave them in the hood for 45min.
4. Aspirate collagen, rinse the dish with 2ml of sterile PBS.
5. Aspirate PBS. Wrap the dishes in foil, label and keep in the cabinet.

B. Cleaning / Autoclaving glassware and instruments (1 or 2 day before the expt).

1. Instruments for dissection

Large scissors, forceps and scalpel - 2 sets. One steel box.

Small scissors, forceps and scalpel - 3 sets. One steel box.

2. Petridish - 8

3. Cell strainer and 200mesh(76µm)

4. 100ml beakers - 4.

5. Coverslips

6. Cotton tip applicators

7. Tips - P-1000, P-200, P-20

Petridish with paraffin - 4- Melt paraffin in each petridish over the hot plate. When cool, leave petridish open under UV overnight for sterilization. Next day wrap them in foil in the hood and store.

Wash steel boxes, instruments and glassware thoroughly with detergent. Wash and rinse with ddH<sub>2</sub>O. Wipe them with 70% alcohol. Wrap each set of instruments in a towel. Put large instruments in one box and small ones in the other. Put the boxes in sterilization pouch and seal. Put a little autoclave tape on each bag.

Assemble cell strainer and put it in a sterilization pouch and seal

Put extra meshes in a sterilization pouch and seal.

Wrap beakers and petridishes individually in foil. Put a little autoclave tape.

C. Preparation on the day of experiment.

Turn on the shaker bath and tissue culture water bath at 37°C. Keep CEMEM, trypsin and drugs in the bath. Put HBSS at 4°C.

D. Sterilization of laminar flow and equipment (1/2 to 1 hr before the Expt).

Wipe the interior surface of hood with 70% alcohol thoroughly. Wipe dissection microscope and pipette-aid with alcohol and put them in the hood. Put the autoclaved instrument boxes, beakers, 50ml tubes and cell-strainer in the hood. Turn on the UV light for 30 min.

## SOLUTIONS FOR PRIMARY ASTROCYTE CULTURE

Collagen - 50 µg/ml in 0.02N acetic acid

PBS, sterile

Complete Eagle's modified essential medium (CEMEM)

Hanks balanced salt solution (HBSS) Ca<sup>++</sup>/Mg<sup>++</sup> free

Trypsin - 0.5mg/ml in HBSS

Trypan Blue

### Collagen Solution 50ug/ml in 0.02N acetic acid

Rat tail Collagen, type A – 3.93mg/ml

Acetic Acid- 17.4N

Take 12.73ml of 3.93mg/ml Collagen

Add 800ml ddH<sub>2</sub>O

Add 1.15ml of 17.4N Acetic Acid

Bring the volume to 1000ml with ddH<sub>2</sub>O

Filter sterilize the solution with 0.2µM filter

### Trypsin

2.5 g/Lit

i.e. 2.5 mg/ml

2.5mg ----- 1ml

0.5 mg -----  $\frac{0.5}{2.5}$  = 0.2 ml

For every 0.2ml trypsin, add 0.8 ml HBSS.

Or

Take 10 ml trypsin and add 40 ml HBSS - 0.5mg /ml

### Complete Eagle's modified essential medium (CEMEM)

780 ml medium

100ml fetal bovine serum

100ml Horse serum

10 ml L-Glutamine

10 ml penicillin/ streptomycin (10,000 µg/ml)

Remove 220-ml medium from 1000ml flask. Add rest of the solutions. Store at 4°C.

### PBS (sterile)

See notebook.

### Dibutyryl cAMP - 0.25mM

TO make **0.25M** stock solution

Add 4 ml sterile PBS to 0.5g dibutyryl cAMP. Mix well.

To make 0.25mM working solution

## **VII. PREPARATION OF ENRICHED OLIGODENDROGLIAL CULTURES FROM RAT SPINAL CORD**

*Modified from Duncan et al (1988)*

### **PROCEDURES**

1. Deeply anaesthetize rat pups (day 7 old, Spray Dawley) in a sealed jar with halothane (2 ml for 1-2 min).
2. Spray the rats with 75% ethanol, wipe the skin of the whole body. Place the animal on a gauze pad. Open the chest with a scissors, poke the left ventricle of the heart with a scalpel blade and the blood is drained.
3. In the hood, pin the rat onto a paraffin dish with the head facing you. Make an incision on the midline of the back skin with a scalpel blade (or a scissors), then separate the skin from the underneath tissue with a new blade and pin the skin on both sides with needles.
4. Trim off the muscles on both sides of the spinal column at the shoulder area. Cut through the spinal column transversely at the base of the skull. Lift the spinal column end with a pair of toothed forceps, then insert one side of the scissors into the column and cut through the vertebrae on both sides horizontally. The cord will be attaching onto the spinal column dissected. Then peel the cord from the vertebrae and transfer it to a petridish with ice-cold L-15 medium (5 ml).
5. Under a dissecting microscope, carefully remove meninges and nerve roots with two pairs of fine forceps (white solid spinal cord fiber vs. soft pinkish meninges membrane). Avoid damaging the spinal cord by grabbing the end or root end.
6. Transfer the cord to a new dish with 7 ml ice-cold EBSS (Eagle's balanced salt solution,  $\text{Ca}^{++}$ ,  $\text{Mg}^{++}$  free) containing 0.25mg/ml trypsin and 50 $\mu\text{g/ml}$  DNase (to make 20 ml, 17.5ml EBSS, 2ml 0.25% trypsin plus 0.5ml 2mg/ml DNase). Split the spinal cord in the center into two pieces with two forceps. Cut the tissue from the top to the end into small fragments ( $<1 \text{ mm}^3$ ) with two pairs of fine sharp blades.

7. To pH the buffer, place the dish in a dessicator and put the dessicator with a cover aside in a CO<sub>2</sub> incubator. Let it equilibrate for 10 min.
8. Incubate the tissue dish in the dessicator at 37°C on a rotating shaker (70 rpm) for 30~45 min.
9. Stop the enzyme reaction by addition of fetal bovine serum (FBS, 1.5ml) to a final concentration of 10%. Transfer the tissue pieces and solution into a 15-ml conical tube with a pipette (rinsed with serum before use!), add 5.0 ml L-15 and then centrifuge at 1000 rpm for 5 min to recover the tissues.
10. Resuspend the tissues with 2 ml of L-15 medium with 10% FBS/50µg/ml DNase (17.5 ml L-15, 0.5ml 2 mg/ml DNase, 2ml FBS), gently triturate the tissues with a P-1000 pipette (set at 800) for x 70. Repeat the triturating with a P-200 pipette (set at 100) until the tissue chunks disappear.
11. Dilute the cell suspension with 5.0 ml L-15 medium/10%FBS/50µg/ml DNase. Add 3 ml of 90% Percoll (9 ml of Percoll + 1 ml of 10x HBSS) and transfer the suspension to a centrifuge tube (an Oakridge 10-ml tube) and mix well with a pipette. Balance the tubes with the adapters, and centrifuge at 30,000g at 4°C for 30 min (Rotor JA20, speed 15,750 rpm, temperature 4°C, Acc 9, Dec 5).
12. Carefully remove the centrifuge tube. There will be 3 bands in the solution. The top white band is mainly debris and myelin and the bottom band is a thin layer of red blood cells. Between the two bands is the clear cell layer and oligodendroglia are usually located in the lower part of the band immediately above the blood cell layer. Remove the first band and upper part of the cell layer with a pipette. Use a new pipette (rinsed with FBS) to transfer the remaining part of the cell layer just above the red blood cell layer to a 15-ml tube. Add L15 / 10% FBS up to 15 ml and centrifuge at 1000 rpm at 4°C for 5 min.
13. Remove the supernatant and resuspend the cell pellet in 2 ml of complete DMEM. To determine the concentration of the suspension, mix 15 µl of cell suspension and 15 µl of trypan blue and place 10 µl of the mixture onto the hemacytometer. Count cells

in the 4 large squares, average the 4 numbers and then multiply by  $2 \times 10^4$ . This is your cell concentration, cells/ml.

14. Cell cultures. Plate the cell suspension onto poly-ornithine coated coverslips in a 24-well plate at a density of 80,000–100,000 cells per coverslip. It is ideal to place 50~100  $\mu$ l of cell suspension onto the coverslip without getting out to the coverslip and let the cells attach to the substrate at 37°C for 10 min before adding 1.0 ml of complete DMEM growth medium.
15. Incubate the cultures in 5% CO<sub>2</sub> at 37°C with a medium change every 3 days. Oligodendroglial cells can be identified by immunocytochemistry with antibodies directed against various antigens that are expressed by cells at different developmental stages.

#### Appendices:

1. Complete DMEM for oligodendroglial differentiation:

DMEM (Gibco)	98.7 ml
N1 mixture (Gibco)	0.5 ml
Insulin (2.5 mg/ml, filter sterile, I-6634 Sigma)	0.2 ml
Biotin (10 mg/ml, filter sterile, B-4501 Sigma)	0.1 ml
FBS	0.5 ml
1ng/ml Human PDGF-AA (1 $\mu$ g/ml, Pepro Tech,#100-13A),	100 $\mu$ l
2. Other solutions
  - Percoll, Sigma P-4937, 1x500ml
  - L-15 medium, Gibco, 11415-064, 500ml/bottle
  - Poly-ornithine, Sigma
  - Trypsin, 2.5mg/ml
  - DNase, Sigma
  - EBSS, Eagle's balanced salt solution
3. Antibodies commonly used for identifying oligodendroglia:

For oligodendroglial progenitors: A2B5 (IgM), anti-PDGFR $\alpha$  (IgG);

For immature oligodendroglia (in 1<sup>st</sup> week of culture): O4 and O1 (IgM), anti-GC (IgG);

For mature oligodendrocytes (in situ or 2 week-old in culture): anti-MBP (IgG), anti-PLP (IgG).

For astrocytes: anti-GFAP (IgG).

#### 4. N<sub>1</sub> Mixture Protocol

	Weight	Solution volume	Further dilution
Apo-transferrin T-0178 (Sigma)	25mg	5ml	
Putrescine P-7505 (Sigma)	80.55mg	5ml	
Progesterone P-0130 (Sigma)	3.145mg	5ml 90% EtOH	50ul to 10ml PBS
Selenium (Na <sub>2</sub> SeO <sub>3</sub> ) S-5261 (Sigma)	5.19mg	100ml PBS	0.5ml to 5ml PBS

#### Final Mixture

Tranferrin            5ml  
Putrescine            5ml  
Progesterone        10ml  
Selenium              5ml  
Total    25ml

Filter with 0.22um filter, Store at -20°C in 1.25ml aliquots 4-6 weeks max.

#### 5. Further reading:

McCarthy KD, de Vellis J (1980): Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. *J Cell Biol*, 85: 890-902.

Raff MC, Miller RH, Noble M (1983): A glial progenitor cell that develops in vitro into an astrocyte or an oligodendrocyte depending on the culture medium. *Nature*, 303: 390-396.

Duncan ID, Hammang JP, Jackson KF, Wood PM, Bunge RP, Langford L (1988): Transplantation of oligodendrocytes and Schwann cells into the spinal cord of the myelin-deficient rat. *J Neurocytol*, 17: 351-360.

Warrington AE, Barbarese E, Pfeiffer SE (1993): Differential myelinogenic capacity of specific developmental stages of the oligodendrocyte lineage upon transplantation into hypomyelinating hosts. *J Neurosci Res*, 34: 1-13.

## VIII. Preparation of Organotypic Hippocampal Slice Cultures Using the Membrane Filter Method

*Modified Bergold and Casaccia-Bonnet (1997) and Stoppini et al. (1991)*

### Procedures

1. Deeply anaesthetize rat pups (day 8-10 old, Spry Dawley) in a sealed jar with halothane (2 ml for 1-2min).
2. Spray the rats with 75% ethanol, wipe the skin of the head with 75% ethanol. Decapitate with large scissors.
3. Make an incision along the midline of the head with a razor blade and peel the skin away to expose the skull. Using small scissors, remove the neck muscles and cut along the midline of the skull from the foramen magnum to the interhemispheric sulcus; follow this with two lateral cuts.
4. Peel away the skull using the needle holder. Invert the head and remove the brain by placing a spatula between the brain and the skull and cutting the cranial nerves and olfactory bulb.
5. Gently drop the brain into a Petri dish containing 1ml of ice-cold GBSS-G (Gey's balanced salt solution supplemented with 6.5g/L glucose and 25mM HEPES).
6. With the scalpel, make two sagittal cuts at the interhemispheric sulcus. Using the large spatula, gently pull away the cortex and the hippocampus from the thalamus and basal ganglia.
7. Using the scalpel, make an incision at the level of the hippocampal fissure. With the small spatula, scoop the hippocampus from the underlying cortex. If the dissection is sufficiently rapid (<10 min), isolate the second hippocampus and slice along with the first hippocampus.
8. Using a sterile wide-bore, plastic transfer pipet, transfer the hippocampi to a polyester film immobilized on the stage of a McIlwain tissue chopper (Brinkman, Westbury, NJ).
9. Align the hippocampus so the temporal end is perpendicular to the chopper blade.

10. Chop rapidly: Approx. 1 chop/sec. Typically, prepare transverse sections of 400-500  $\mu\text{m}$ .
11. Using a sterile wide-bore, plastic transfer pipet, transfer sliced hippocampi to a 35-mm dish containing 5ml of ice-cold GBSS-G and separate the slices by shaking the dish or by use of a fine paint brush. **Use great care** since the slices are easily damaged. Incubate the separated slices for 1h at 4°C.
12. Place the sliced hippocampus under a dissection microscope. Select slices having even margins and clear, uniform, well-defined pyramidal cell layers to be plated on membrane inserts.
13. Pipet 1 ml of SCM (slice culture medium) into each well of a six-well culture dish. Place a Milli-Cell CM filter insert into each well.
14. Using a sterile wide-bore, plastic transfer pipet, transfer 1-3 slices to the wet surface of the filter insert and remove excess fluid using a micropipet. Place the slices close to the center of the insert, but keep slices separated by more than 2 mm.
15. Maintain cultures at 35°C in a humidified 95% air, 5% CO<sub>2</sub> atmosphere. Completely replace the SCM every 3-5d.
16. After 4-5 d in vitro (DIV), remove actively mitotic cells by treatment with anti-mitotic medium for 24 h (100 nM 5'-flurodeoxyuridine, 100 nM cytosine arabinoside and 100nM uridine in SCM). Refeed slices with 1ml fresh SCM in each well.
17. Examine the cultures after 10-12 DIV to check the preservation of pyramidal and granule cell layers. Discard cultures that display selective neuronal loss of a hippocampal region. Cultures may be used up to 28 DIV.

Appendices:

1. Complete SCM (1000ml):

50% MEM (Gibco)	500ml
25% heat-inactivated horse serum (Gibco)	250 ml
25% Eagle's balanced salt solution (Gibco)	250 ml
Glutamine 1mM	

Glucose 6.5g

Sodium HEPES 25mM, pH 7.2.

## 2. References

- P. J. Bergold and P. Casaccia-Bonofil (1997)

*Methods in molecular Biology, Vol. 72: Neurotransmitter methods, page 15-22.*

- L. Stoppini et al. (1991)

*A SIMPLE METHOD FOR ORGANOTYPIC CUTURES OF NERVOUS TISSUE.  
J. Neuroscience methods 37:173-182.*

## IX. CELL DAMAGE ASSESSMENT BY CALCEIN-AM/PROPIDIUM IODIDE

### SOLUTIONS

#### Calcein-AM-Stock Solution (0.5mM)

Dissolve 50 $\mu$ g Calcein-AM in 100 $\mu$ l DMSO

#### Propidium Iodide - Stock Solution (5mg/ml)

Dissolve 10mg PI in 2ml PBS

#### Calcein-AM/PI -Working Solution (2.5 $\mu$ M)

12ml Isohepes + 30 $\mu$ l of 0.5mM Calcein-AM + 12 $\mu$ l of 5mg/ml Propidium Iodide

### PROCEDURE

1. Wash cells with isohepes. Add 1.5 ml fresh medium containing 2.5 $\mu$ M Calcein-Am and 5 $\mu$ g/ml PI.
2. Incubate at 37°C for 30min.
3. Wash with 2 ml isohepes just once, then add exactly 1 ml of isohepes.
4. Observe under fluorescence microscope (FITC filter for Calcein-AM and TRITC or rhodamine filter for PI)
5. Count Calcein positive and PI positive cells in at least 2 fields for each treatment as well as controls

### Operating Fluorescent Microscope

Turn lamp on. **Make sure that the computer is off.**

Turn the computer on (with the red switch on the power strip).

Turn light button on.

Turn the knob on the right side of microscope to A to view the plate.

After focussing, turn the knob to C (CCD camera).

Click metamorph program on the computer.

Click open the fluorescence and then click shutter button on the tool bar at the top of the screen.

Click 100ms/200ms/500ms depending on the exposure time needed and take a phase contrast picture

Inset the FITC/TRITC filter and take pictures.

Change the picture to desired color by clicking circle on the left of the window.

Convert the picture to 8 bits.

Save as TIFF file on zip disk (Zip drive) or on CD.

### Counting live/dead cells

Click measure on the tool bar.

Click manual counting

Open file and click individual cells and record the number from the chart at the end.

## **X. Suggestion on form for lab notebooks**

**a. Record Keeping:** a spiral notebook (avoid using loose paper).

Name of author

Date of experiment

Number and name of experiment: copy from manual

Purpose: short description

Equipment used: manufacturer's name and serial number

Procedure: follow the manual and indicate differences from the suggested procedures

Calculations: give formulae used

Original data: must always go directly into your notebook.

Errors: cross over instead of erase them

Data and results: at the end of an experiment, write some comments and summary of experiments and results

**b. Partners:** all partners should record the experiments and turn in reports independently

**c. Completion of work:** Don't take your notebook home to "write it up". Try to complete your notebook before you leave the laboratory.