

## TISSUE CULTURE

### I. Chick Retina Cultures

#### Preparation of TC dishes

- Add 2ml of 1XPORN (0.05 mg/ml polyornithine) solution to each tissue culture (TC) quality 35 mm dish for 3 hours in hood.
- Wash with sterile ddH<sub>2</sub>O one time \* (at this point dishes can be stored in 4°C for extended periods of time)
- Add 2ml's 199 medium with pen-glu in incubator overnight
- Remove media
- Add 1ml 2X PhR media immediately preceding dissection and keep in incubator until ready to seed dishes (you eventually add 1 ml of cell suspension in 199)  
\*\*\* E6 chick retinas yield ~ \_\_\_\_\_ cells per ml,  
E8 chick retinas yield ~ \_\_\_\_\_ cells per ml.

#### Preparation of glass coverslips on which to grow cells

- Circular #1.5 coverslips (ideal for confocal microscopy since lenses are corrected for this thickness) from Corning are degreased in acetone and ethanol for 20 minutes each on a shaker.
- Etch for 1 hour in 1M HCl (others use 1N NaOH or nitric acid) while shaking, and wash by shaking in large volume of sterile water overnight. Sterilize by baking in oven.
- When ready to use coat with 1xPORN for at least three hours, wash once in sterile water.
- Incubate O/N in 199 medium
- Add 2xPhR media prior to dissection.

#### Dissection

- Perform dissection in 1X HBSS in 100mm bacterial grade dishes (TC grade dishes are much more expensive and should not be used for this step!)
- Transfer retina to 1X CMF and cut into small pieces with tungsten needles
- Using siliconized pipet, transfer small retina pieces to white capped tube with CMF and incubate in water bath for 10 minutes
- While tissue is incubating:
  - 1- Remove 1 trypsin aliquot and place in water bath
  - 2- Remove 1 Dnase aliquot to thaw at room temperature
  - 3- Set up 2 60mm Petri dishes in hood, one marked "W" for waste
  - 4- Set up 8 siliconized, plugged pastuer pipets on stand
- When trypsin and Dnase are thawed, mix the two together with a pipet
- Take out of bath and remove solution without disturbing tissue at bottom of tube
- Add all of the Dnase/Trypsin solution, keeping tissue at bottom
- Put in water bath for 20 minutes
- During 20 minute incubation:
  1. flame bores of three siliconized, plugged pippets to reduce bore size
  2. Add 25 mls LEBM/-1%BSA to 1 petri dish
  3. Fill 50ml, red capped tube with 199 pen/glu: 5ml for each E8 eye, 2ml for each E6 eye
  4. Clean and set up hemacytometer with coverslip
- After 20 minutes, add 3 pipet fulls of LEBM-1% BSA (without removing Trypsin/Dnase) to tube
- Take out all media, being careful not to disturb tissue at bottom of tube

- Add 3 more pipet fulls of LEBM-1%BSA and remove
- Add 1 more pipet full and remove most of media
- Take smaller bore pipet and fill with LEBM-1%BSA
- Put pipet on bottom of tube and dissociate 20X, being careful not get air bubbles or foam
- Put Nytex filter (or Falcon cell strainer) in 50ml tube, over 199 solution and wet with some 199
- Use pipet to take out cell solution and pass through filter
- Wash tube again, not to lose cells
- Take out a little cell solution and fill hemacytometer to count cells.
- Count cells in 5 fields-four corners and center square. Count both sides of hemacytometer, take average.

~800,000 cells should be plated onto each 35mm dish (this is a low density culture)

Notes on the dissection procedure:

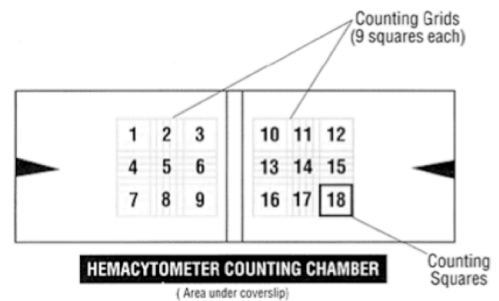
- A. Never let the tissues dry out during dissection. Always keep them immersed in a balanced salt solution. Cells will be damaged and tend to lose different ions if not protected by a physiological solution.
- B. The presence of protein in the dissecting buffer will also protect the cells. Chick serum, which does not contain any trypsin inhibitors, can be used at 5-10% concentration. Do not use sera rich in trypsin inhibitors, such as horse serum, because they can complicate the dissociation.
- C. Reduce mechanical trauma to an absolute minimum.
  1. Teasing tissues apart is a very damaging procedure.
  2. Cutting tissues with very sharp instruments is much less traumatic than teasing. Cutting breaks only cells directly adjacent to the instruments, while teasing breaks many more cells, including those not directly exposed to the dissecting instruments.
  3. For cutting, a tungsten needle in one hand and an iridectomy knife in the other make a good combination, provided they are sharp and that you use them for cutting and not teasing. You can also use needle making a scissors motion with them, or a needle and a pair of iridectomy scissors.
  4. A Pasteur pipet "charged" with dissecting medium is more appropriate and less traumatic than forceps for transferring tissues from one dish to another. However, you must use pipets carefully; charge the pipet with dissecting medium, introduce the tip into the solution containing the tissue, expel a small amount of buffer, and take the tissue and release the bulb completely before taking it out of solution. This procedure prevents bubble formation. If you are transferring tissue to a solid substrate (agar, plasma clot, etc) you can control the position of the tissue within the pipet (close the mouth) thus reducing the amount of fluid that you add to the substrate.
- D. If you have to dissect more than one animal and pool the tissues, there is no way to have all the tissues treated in exactly the same way – either some will wait longer before being taken out of the animal, or they will spend more time in BSS after being taken out of the animal. The procedure must be standardized. This problem must be considered in the design of any experiment.
- E. Sometimes a concave surface is more practical than a flat one, especially if you have to collect small organs such as ganglia. A depression slide or a watch glass contained within a Petri dish can be used.

\* suggested working volumes \*

| <b>Volumes:</b>    | 35mm            | 60mm              | 100mm           | 24 well         |
|--------------------|-----------------|-------------------|-----------------|-----------------|
| PORN               | 1.5 ml          | 4.0 ml            | 8.0 ml          | 1.0 ml/well     |
| ddH2O              | 2.0 ml          | 5.0 ml            | 9.0 ml          | 1.5 ml/well     |
| 199 pen-glu        | 1.5 ml          | 4.0 ml            | 8.0 ml          | 1.0 ml/well     |
| 2X PhR media       | 1.0 ml          | 3.0 ml            | 7.5 ml          | 0.5 ml/well     |
| Seeding soln       | 1.0 ml          | 3.0 ml            | 7.5 ml          | 0.5 ml/well     |
| Total final volume | 2.0 ml          | 6.0 ml            | 15 ml           | 1.0 ml/well     |
| <b>Densities:</b>  |                 |                   |                 |                 |
| Seeding cells/ml   | $8 \times 10^5$ | $8 \times 10^5$   | $8 \times 10^5$ | $4 \times 10^5$ |
| Final cells/dish   | $8 \times 10^5$ | $2.4 \times 10^6$ | $6 \times 10^6$ | $2 \times 10^5$ |
| Final cells/ml     | $4 \times 10^5$ | $4 \times 10^5$   | $4 \times 10^5$ | $2 \times 10^5$ |

### Cell Counting – how to use a hemacytometer

- Load one side of hemacytometer with a cell suspension.
- Count at least two fields (indicated in gray).
- Since each field is  $10^{-4}$  ml, divide the total counted by the number of squares counted and multiply by  $10^4$  to give cells per ml. In other words, if you count 70 cells in a single grey box you have 700,000 cells per ml.



### Cell Viability – Trypan Blue staining

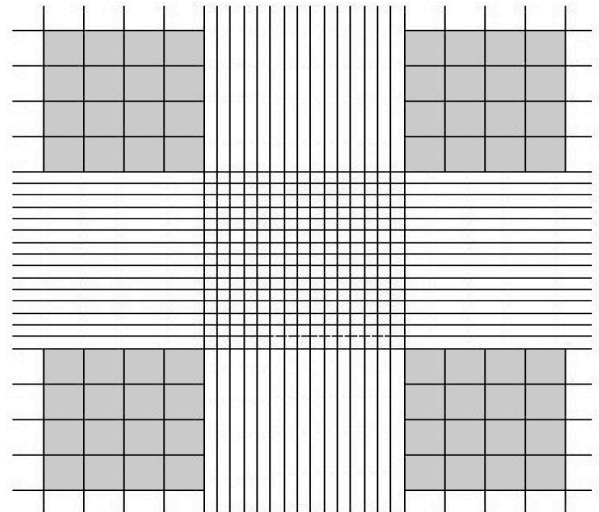
The use of Trypan Blue (0.4g Trypan in 100ml HBSS) as a viability indicator is based on the ability of live intact cells (ie-viable cells) to exclude the dye.

#### Gibco directions:

1. place 0.5ml cell suspension ( $1-2 \times 10^5$  cells/ml) in a tube.
2. Add 0.1 ml of the 0.4% Trypan Blue and mix.
3. Allow to stand about 5 minutes, but not over 15 minutes.
4. Fill hemacytomter.
5. Make a total cell count and count of unstained cells.
6. Assuming unstained cells to be viable, express the results as % viable cells
7. Solution is 1/1.2 dilution of the cell suspension. Therefore multiply by 1.2.

#### Alternate Procedure:

1. Mix 0.2 ml cell suspension with 1.8 ml saline or media.
2. Add 1.0 ml of the 0.4% Trypan Blue Stain.
3. Incubate 15 minutes at 37°C.
4. Read in hemacytometer: dead cells stain dark blue, living cells do not.
5. When calculating, remember that the counting solution is a 1/15 dilution of the cell suspension. Therefore multiply by 15



$$\% \text{ Viable cells} = \frac{(\text{Non-stained cells})}{(\text{Total Cells})} * 100$$

## **II. Chick Müller “Glia” Cultures**

1. Prepare culture dishes as for regular dissection, except there is no need to coat with polyornithine.
2. Dissect 8 day chick NR as usual, but seed at a 3X density. Use PhR medium.
3. (optional). Add 1ml PhR (1X) to each 35mm dish on day 3 or day 4.
4. Weekly: Wash dishes with Hank's or 199 medium, replacing medium with fresh 1x PhR.
5. Flat cells can reach confluence within 2 weeks, neuronal elements die by week 3.

## **III. Chick Embryo RPE Cultures**

1. Place embryo in petri dish with KHBSS\* (sterile). Remove eyes and place in fresh Petri dish with BSS.
2. Trim eyelids and some muscle and connective tissue from eyes, leaving some tissue for grasping with forceps during later dissection.
3. Remove anterior portion, lens, and vitreous. Do not leave any iris tissue.
4. Cut eye wall into 4-8 segments, depending on stage of embryo. Transfer these (still with neural retina) to a fresh sterile KHBSS without Ca<sup>++</sup> or Mg<sup>++</sup>. Incubate at room temperature or 37°C, for about 10 minutes.
5. Dissect neural retina away from RPE. Remove pectin.
6. Incubate schlera-choroid-RPE in KHBSS without Ca<sup>++</sup>, Mg<sup>++</sup>, plus 4mM EDTA at 37°C for about 10 minutes.
7. Using five jewelers' forceps, dissect sheets of RPE away from Bruch's membrane. You may want to pin down the eyewall, using fine insect pins, onto wax.
8. Collect RPE sheets in a minimal volume and transfer either to growth medium or to enzyme solution for 3 minutes (dispase or trypsin). If early enough embryo is used, the sheets may break up mechanically by pipetting into small clumps of cells. Plate in growth medium. (We use DMEM + 5%FBS ± 0.5% chick embryo extracts.

- KHBSS – Konigsberg's Hank's BSS complete, with glucose and bicarbonate, pH7.2

### ORDERING INFO

| Catalog # | Company | Description | Size | Contact |
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