Culturing Chick Embryonic Myoblasts/Myotubes and Fibroblasts

Day 1

Materials

1. 100 ug/ml gelatin (described separately later).

Procedure

1. Pipet 10 ml gelatin onto each of two 100 mm culture dishes. Leave in sterile hood overnight.

Day 2

Materials

1. Culture medium: Eagle's MEM with (exactly) 10% horse serum, 2% embryo extract, 100 units/ml penicillin, 100 ug/ml streptomycin, on ice.

2. Saline G, on ice.

3. Sterile distilled H_2O.

4. Sterile 15 ml conical centrifuge tubes.

5. Dissection tools: 2 pairs of coarse forceps, 2 pairs of fine forceps, 1 pair of large scissors, 2 pairs of fine scissors, 1 pair of angular fine scissors. Soak in 95% EtOH and air dry in hood before use.

6. Egg carton.

7. Ethanol (70% in a spray bottle and 95%).

8. Two sets of sterile glass petri dishes, sitting on ice in a plastic pan.

9. Two petri dishes with gelatin solution, prepared on day 1.

10. Nitex filter (20 um), preassembled by tying a piece of nitex around the holder with string and autoclaved in a glass can.

11. 6 ml plastic syringe.

12. 11-day eggs (3 embryos provide enough cells for 10 injection dishes).

Procedure

1. Remove gelatin solution from two petri dishes. Rinse plates 2x, each with 10 ml sterile distilled H2O. Make sure plates are completely dry after rinsing.

2. Add 5 ml medium onto each plate. Place in 34^{o}C incubator.

3. Set up a 'Preplate' by adding 5 ml medium to an untreated 100 mm culture dish. Place in 34^{o}C incubator.

4. Place 2 ml medium in a sterile 15 ml centrifuge tube and let it sit on ice.

5. Put ~2 ml Saline G in one of the chilled sterile petri dish.

6. Sterilize 6 eggs: spray the top (blunt) surface 2x with 70% EtOH, let them air dry between the two sprays. Then wipe with 95% EtOH using Kimwipe. Let dry.

7. Punch a hole on the shell with the large scissors and cut around the top surface. Remove the embryo by holding the neck with forceps. One pair of forcep in each hand will be useful. Collect up to 7 embryos on the chilled petri dish without medium.

8. Cut head off with a pair of fine scissors. Collect heads in a disposable petri dish and dispose.

9. Remove, or pull off skin from the thigh muscle. Hold the distal end of the leg with one forcep. Peel skin off and fold on the body.

10. Dissect the upper thigh muscle close to the body. Make small cuts on the muscle near the joints with a pair of fine scissors and pull off muslce with fine forceps. Do not go above the upper joint or lots of fibroblasts will enter the culture. If desirable, breast muscles can be collected by removing skin from the chest and picking muscles above the rib cage and between two wings.

11. Collect muscle in the petri dish with cold saline G.

12. Rinse muscle 2-3x with ice cold saline G to remove RBC. Use a sterile Pasteur pipet. Remove any apparent pieces of skin and bones. Remove ~40% of the fluid after each rinse. The fluid should be clear (not pink) at the end of this step. The dish may be held out of the ice for a short while if necessary.

13. Mince muscle in a small volume of saline G until uniform pieces are obtained. Keep the dish on ice.

14. Transfer the minced muscle to 2 ml medium in a sterile centrifuge tube (step 4). Use sterile Pasteur pipet.

15. Vortex 30 sec at full speed. Allow pieces to settle and then pipet supernatant to a nitex filter apparatus. Keep the tube with pieces on ice.

16. Filter supernatant through 20 um nitex filter. Gently apply negative pressure with a 6 ml plastic syringe. Hold the filter at an angle so that filtrate can flow along the side of the tube. Keep the collecting tube on ice.

17. Add 2 ml cold medium to settled pieces. The medium can be used to rinse out the petri dish before adding to the tube.

18. Vortex 1/2 speed for 15 sec.

19. Filter supernatant through same filter.

20. Get 'Preplate' from the incubator. Spread the filtrate on the plate.

21. Incubate 25 min in the 34^{o}C incubator.

22. After incubation, divide supernatant into two halves and add them to the two gelatin-coated plates. Put in the 34^{o}C incubator for 24 hr.

23. Prepare collagen-coated plates/dishes as described separately later. Need 16 plates.

Day 3

Materials

1. Saline G, in 34^{o}C water bath.

2. Soybean trypsin inhibitor solid.

3. Medium, as for day 2, on ice.

4. 3x crystallized trypsin in saline G, on ice.

5. Millex-GV filter and syringe.

6. 15 ml conical centrifuge tubes.

Procedure

1. Finish the preparation of collagen-coated dishes as described later.

2. Measure 1-2 mg soybean trypsin inhibitor. Dissolve in cold medium at a concentration of 0.5 mg/ml.

3. Sterilize trypsin inhibitor with the Millex-GV filter. Filter into 15 ml conical centrifuge tubes. Each tube contains 0.5 ml and will be used for one gelatin-coated plate. Keep them on ice.

4. Check the primary culture on gelatin-coated dishes. There should be many single cells and little fusion. Aspirate off medium.

5. Rinse dishes with 10 ml warm saline G. Add the saline G slowly at the edge.

6. Remove saline G completely. Add 5 ml cold trypsin to each dish. Let it sit at room temperature and swirl occationally.

7. Check cells under the microscope. Within 2-5 min, about 50% of myoblasts should detach and the other 50% become round, while most fibroblasts should remain attached to the dish.

8. Wash myoblasts off the dish by tilting dish 45o and drip medium near the upper side of the dish with a Pasteur pipet. Rotate dish 90o and repeat the process.

9. Transfer solution to the tube with cold soybean trypsin inhibitor. Mix by pipeting.

10. Count cells with hemocytometer. Keep the tube on ice. Cell number per ml = number in 25 small squares x 104.

11. Dilute if necessary. Plate cells onto collagen-coated dishes.

Number per dish = 4 x 103; 8 x 103; 1.2 x 104; 1.6 x 104.

Perform serial dilution with cold medium if necessary. Mix well and keep the suspension cold. The volume added to each chamber dish should be 0.1-0.3 ml. Mix the suspension well and use glass 1 or 2 ml pipet to remove cells. Innoculate quickly to avoid cells settling.

12. Swirl dish to spread cells out and place in 39oC incubator.

Day 4

Feed with 2 ml fresh medium per chamber dish.

Day 5 on

1. Examine plates.

2. Apply ara C if there is apparent fusion, i.e. presence of myotubes with ~10 nucles.

3. Afterwards replace medium every third day (no ara c).

Ara C Stock (1 mM)

1. Add 1 mg ara c to 2.5 ml distilled H2O.

2. Sterile filter through Millex-GV filter.

3. Store in freezer (-20^{o}C).

4. Use at 5 x 10-6 M in regular skeletal muscle medium (100 ul ara c in 20 ml medium or pipet 10 ul directly onto chamber dish with 2 ml medium).

Gelatin Stock (100 ug/ml)

1. Add 10 mg gelatin to 100 ml distilled H2O in a medium bottle.

2. Sterilze by autoclaving for 20 min (slow exhaust), mix thoroughly.

3. Store at 4^{o}C (stable for months).

Coating Chamber Dish with Collagen

Materials

1. Soluble calf skin collagen (Cooper LS01660), diluted 1:100 with sterile distilled H2O (1 ml + 100 ml) and stored at 4^{o}C.

2. 3.76 M sterile NaCl (filtered with 0.22 um filter).

3. Chamber dishes, sterile.

Procedure (perform in sterile hood)

1. Pipet diluted collagen solution into a sterile flask (need 1.5 ml collagen per chamber dish).

2. Add 40 ul 3.76M sterile NaCl per ml collagen. Mix gently.

3. Plate collagen solution onto chamber dishes.

4. Let sit overnight under UV light in the sterile hood with lids partially off.

5. Aspirate off collagen solution.

6. Rinse 2x with sterile distilled H2O. Remove all H2O. Do not scratch the coated surface of the dish.

7. Pipet 1.5 ml desired medium onto dishes and put in the 39^{o}C incubator.

CHICK SKELETAL MUSCLE FIBROBLASTS

Materials

1. F12K with glutamine, 10% FCS, 1% penicillin/streptomycin.

2. 100 mm tissue culture dish with 5 ml medium, incubated at 34^{o}C.

3. Sterile 15 ml conical tube with 0.5 ml soybean trypsin inhibitor. Prepared in the same way as for myotube culture, Day 3.>

4. 3 x crystylized trypsin in saline G, thaw and keep on ice.

5. STE, in 34^{o}C water bath.

6. 'Preplate' from myotube culture, Day 2 step 22.

Procedure

1. Rinse preplate with 10 ml STE.

2. Apply 5 ml cold 3x crystalized trypsin. Swirl and wait until 50% of cells have detached.

3. Pipet trypsin onto cells to remove more cells.

4. Pipet the suspension into the tube with 0.5 ml soybean trypsin inhibitor.

5. Plate onto the dish with 5 ml warm F12K medium.
