

Starting primary cell cultures from tissue explants

Fetal Ligament (bovine): FCL cells.

1. Remove ligament from animal using as sterile technique as possible. A one inch segment from an older 270 day old fetus will be more than sufficient. For the younger fetus (100 days --- 150 days). You will want to remove as much as possible. Place the “sterile” tissue in a sterile bottle containing Hanks balanced salt solution with penicillin/streptomycin added. Working in a sterile hood, cut the ligament into long thin strands or noodles, then mince each slice into as small pieces as possible (less than .5 mm).
2. Using a pipet, add media to the sterile plate upon which the ligament was chopped and pull the mixture of tissue and medium up into the pipet. Dispense a generous, equal amount of tissue into separate T 75's. Using a circular motion, disperse the tissue pieces onto the bottom of the T flask. Then tip the flask up on its side, loosen the cap by 1/4 turn to allow CO₂ atmosphere exchange, and let tissue “dry” onto the bottom of the flask for 1-2 hours. Gently lay the flask back down, allowing media to again surround and cover the tissue.
3. Cells should begin growing out within about five days. The younger cells (100) grow extremely quickly and should be passed before they begin to clump into small balls of cells. If this does happen, filter out the cell clumps. The 270 cells will usually be ready to subculture in 2 1/2 to 3 weeks. Begin initial feeding of the primary explant cultures when the cells are well established – this usually is 5-7 days after the initiation of the culture.

Chondrocytes.

1. Also removed from fetal bovine using sterile technique. We have found it important to obtain ears only from a fetus removed directly from the amniotic sack. The best age of ears to use seems to be about 150 days old – the older ages take far too long to digest. Again the ears are placed in sterile solutions.
2. Back at the lab, strip away the covering skin to obtain the cartilaginous inner material. Cut off all the edges and the front of the ear where the ear would be attached to the head. Scrape all over the surface with a scalpel to remove any fibroblast containing tissue.
3. Mince the cleaned cartilage material into small .5 – 1 mm pieces. Add the pieces to a sterile solution of digestion buffer in a small media bottle. Shake on an orbital shaker at 37°C until the tissue minces are about 3/4 dissolved.

DIGESTION BUFFER:

.01 M calcium acetate
.05 M Tris pH 7.4
.15 M NaCl
2 mg/ml collagenase

4. Filter the solution through several layers of sterile gauze into a culture tube. Centrifuge 10 min. x 1000 rpm. Resuspend cells in 10ml FCS DME P/S NEAA. If there still seems to be a lot of clumps of debris you may want to filter and recentrifuge again. Do a cell count using a hemocytometer --- do not use a coulter counter. There will be so much debris, lysed cells etc. that the coulter counter will give an artificially high value. When counting in the hemocytometer be sure to count only viable cells. These cells are very small and should have a refractive halo-type appearance to them if they are viable. Plate at approximately 10,000,000 cells/P100 or the same relative density for other sized dishes.

Skin fibroblasts.

1. The sterile skin should be scraped exhaustively on the under side to remove as much fat as possible. Again, the smaller the pieces the better the cells appear to grow out.
2. Disperse and aliquot out as for ligament. On secure under sterile glass coverslips with sterile silicon grease.
3. These cells will take far longer to grow out (1-2 months).

Maintaining Cells in Culture

A. Feed cells biweekly.

Remove media with sterile pasteur pipets. For primary cultures try not to remove the tissue minces. At 2 weeks the tissue minces can be removed and replated on new T-flasks for cells to be used for membrane study work. Be sure to note B-2 for this replated tissues so they will not be used for elastin studies.

Note: Feed spinner flasks with 30mM Hepes, DME (HG/LB), 10% FCS (100438), P/S, NEAA.

B. Subculturing Cells

Cells should not be allowed to get overly confluent. This occurs when cells reach confluence and are not passed after approximately 1-2 weeks. A heavy layer of cells with much extracellular matrix will develop. When trypsinized the cells will not break down to single cells, but to sheets or clumps of cells.

Wash with CMF (calcium-magnesium free) ECS. Add 5 ml/P100 of 0.5% trypsin-0.02% EDTA. Incubate cells and trypsin 5-10 min. at 37°C. Remove cells and trypsin solution to sterile conical culture tube, adding an equal volume of 10% ECS DME etc., to inactivate trypsin. Centrifuge 10 min x 1000 rpm. Wash cells in EBS 1X and recentrifuge as above. Resuspend cell pellet in appropriate medium and plate out at $1-1.5 \times 10^6$ cells/P100 or the relative equivalence for different sizes of tissue culture wall. Cell should be attached within 4 hours.

Plate cells at these approximate densities:

FCL cells:

1,000,000 to 1,500,000 cells / P-100
10,000,000 to 15,000,000 cells /large roller bottle
7,000,000 to 8,000,000 cells/small roller bottle
10,000 to 20,000 cells/ IF well

FBC cells:

10,000,000 cells/P-100
100,000,000 cells/large roller bottle
50,000,000 cells/small roller bottle
50,000 cells/IF well

C. Freezing Cells

Prepare cells exactly as for subculturing. (Wash with CMF EBS, trypsinize, centrifuge, wash, recentrifuge). Suspend cells in 95% FCS 5% DMSO. DMSO should be filtered through a sterile CR acrodisc filter. Gently disperse cells in FCS-DMSO solution. Final concentration should be approximately 1×10^6 cells/.75ml. Dispense cells with a 5cc or 10cc syringe into sterile 1 ml Nunc cryovials (#3.66656). Screw lids down until just snug, do not overtighten or silicon seal will break. Label cryovials with cryomarkers. Indicated cell name, pass number, #cells/vial, and date frozen.

Store in a double layered orange styrofoam container in which 15ml sterile culture tubes arrive. First acclimate cells to -70°C overnight. Next day, put into liquid nitrogen vessel, preferably in the lower 3 pies. Be sure to note where cells are being filed onto the spiral notebook and log into the computer.

D. Thawing and Plating Frozen Cells

Remove vial of cells and thaw rapidly in 37°C both. (Note on spiral notebook and computer space being filed). Remove cells gently, dispersing first to make sure to remove settled cells. Add cells to regular cell culture medium. As soon as cells are attached (approximately 4 hours) change medium to fresh cell culture medium to remove DMSO. Feed and subculture as you would normally.

F. Culturing Cells on Cytodex Beads:

Preparation of Cytodex Beads:

There are 3 types of Cytodex (I, II, III). Cytodex 2 is the best for our usual cell types. Swell (1 g/100ml) for 3 hours in PBS. Discard PBS and wash with gentle agitation for a few minutes in fresh PBS (50 m/g cytodex). Discard PBS and replace with fresh PBS (50 ml/g). Sterilize by autoclaving (115°C, 15 psi, 20 min).

Prep of beads for cells:

Remove desired amount of beads and allow to settle for several minutes, eventually removing PBS and replacing with cell culture medium. If desired, you can repeat this procedure. Note how many beads are used (g/ml).

Prepare cells as usual and get a cell count. For a cell culture with a final column of 100ml, add 400mg sterile cytodex to a 100ml spinner flask. Add approximately 30ml culture medium and about 5×10^7 cells. Incubate at 37°C and spin the mixture for about 10 seconds at low speed. Allow the cells and beads to incubate without stirring for 30 minutes. Stir the mix again for 10 seconds. Repeat this procedure for about 3 hours. (For cells with a tendency to aggregate, slow (10 rpm) continuous stirring is required. Once the cells have flattened onto the microcarriers, bring total volume to 100 ml and stir at a speed which just keeps the microcarriers in suspension.

Medium:

10% FCS
DME (HG/LB)
P/S
NEAA
30mM Hepes

Depending upon inoculating density, microcarrier mix will probably not need to be fed for 4-5 days. As cells become more confluent, feed daily replacing 30% of medium each day.

Staining

Remove a small drop of cell-bead suspension onto a glass slide. Add one or two drops of 1% methylene blue and gently mix. Cover with cover slip.

G. Prep of Solutions for Cell Work

1. Trypsin – EDTA
Make 10X solution of:
0.5g Trypsin
0.2g EDTA
EBS (CMF)

Filter sterilize 1X solution. When making 1X, bring up volume with EBS (CMF) or HBS (CMF) and filter sterilize.

Add fresh media as follows:

6ml/P100

10ml/T75

3-4ml/P60

2ml/P35

1ml/24 well plate

30ml/490cm² roller bottle (small)

60ml/800cm² roller bottle (large)

2. All cells in upper part of incubator (non-roller bottle) should be fed with 10% FCS DME (HG/HB) P/S NEAA

Use only 10% FCS (be sure to use a serum that has been lot tested for acceptable growth of cells and elastin production) .

3. Roller bottles should be fed with:

5% FCS

5% Calf Serum

30mM Hepes DME (HG?LB) p/s NEAA

When there are primary cultures, do not feed them until cells are growing out fairly well (usually one week or so).

4. Spinner Flasks

When cells are well established, daily remove approximately 30% of the medium and replace with 30% fresh medium. Use same medium as for roller bottles. On Fridays, remove nearly all medium and replace with fresh to get through the weekend. Store at -20°C in 50-100ml aliquots. Do not freeze-thaw repeatedly, activity is lost.