MEI Protocol

CHICK EMBRYO MUSCLE CULTURE PROCEDURE

SETTING UP

1. Turn on blower in hood.
2. Warm-up bottle of Puck's and one of medium.
3. Put 3 pr. of forceps, scissors, and 2 magnetic stir bars into alcohol bath.
4. Remove eggs from incubator.
5. Wipe down hood with 70% alcohol.
6. Place 2 large (100mm x 20mm) dishes into hood.

PREPARATION

1. Wash eggs with alcohol.
2. Open up petri dishes, take instruments and one stir bar out of alcohol bath and place the stir bar into petri lid and forceps and scissors on the edge.
3. Wash eggs a second time with alcohol.
4. Pour 60 ml of Puck's into one of the 100 ml bottles.
5. When eggs are dry begin dissection.

REMOVE EMBRYO

1. Tap cop of egg with forceps with enough force to make hole in egg.
2. Peel off top of egg by cutting around the top with forceps.
3. Using a different sterile forcep, peel back inner membrane and pull embryo out by slipping forceps under the neck and gently lifting out
4. Place embryo into petri bottom.

Repeat above procedure until the desired numbers of embryos have been obtained.
For a desired cell concentration of 1.4×10^5 cells/ml:
Approximations: 3 embryos- up to 100 35 mm dishes
10 embryos- up to 200 35 mm dishes

CLEAN EMBRYOS

1. Wash embryos with approximately 10 ml of Puck's solution. Rinse embryos several times by swirling dish to adequately rinse blood
2. Suck off Puck's with unplugged pasteur pipet
3. Rinse again with an additional 10 ml of Puck's

DISSECTION

1. Remove head and place back into egg shell.
2. Using two pair of forceps, (not the one used to open the egg), remove the arms and lower leg and foot- place in top of second petri dish.
3. Start with embryo on its stomach.
4. Using both prs. of forceps, remove the tail and then remove skin from the back of the embryo.
5. Peel skin away from the thigh muscle.
6. Turn embryo over and remove skin from the other side of the leg. There shouldn't be much skin left on this side because a lot of the skin is usually removed while doing the other side. Any skin that may be left tends to remain around the abdominal region.
7. Flip the embryo back over onto its stomach and check again for skin left on that side.
8. Using forceps, separate the lag from the middle of the back below the pelvic bone.
9. Follow the above procedure for removing the thigh muscles from the remaining embryos. Place the legs that are removed into a new large sterile petri dish with approximately 20 ml of Puck's in it
10. Shake legs with forceps to remove any loose skin-suck off Puck's in dish and add approx. 20 ml new Puck's

REMOVE BONES

1. Remove bones by gently pulling tissue away from the bones. Use one pair of forceps to hold the bone, the other pair to pull away the tissue. Place the bones in the second petri dish top with the other disgarded tissue.
2. Place a tube of trypsin into lukewarm water to thaw.(Trypsin is used at 2 ml for 50 ml of Puck's and tissue. Trypsin = 0.25% Gibco cat. # 610-5055 and it is made up in 50 ml of Puck's.)
3. After all the bone has been removed from the muscle tissue again tease through the tissue to make sure that there are no small pieces of bone left. Any pieces of bone that are left will later clog the pipets.
4. Now cut the tissue with the scissors until the tissue is cut into small pieces.
5. Pour the tissue and Puck's into a 100 ml sterile bottle.
6. Add the thawed trypsin to the bottle. Drop a dried magnetic stir bar into the bottle. (Add Puck's as needed to bring the total volume up to 50 ml).

SPIN MIXTURE

1. Place the 100 ml bottle onto magnetic stir plate and stir for 15 minutes.
2. During this 15 minutes period, remove any trash from the hood. Save the sterile petri dish lid and put it to the side. Scissors and magnetic forceps go into the trypsin water to soak and the demagnetized forceps go back into the alcohol.

3. Also during this time place sterile water onto the 35 mm dishes (1.5 ml) and into the 24-well plates (0.5 ml/well) and place back into the incubator.

CENTRIFUGATION

1. After 15 minutes, remove mixture from the stir plate.
2. Add 10 ml of medium to mixture and then evenly distribute mixture between two 50 ml centrifuge tubes.
3. Place these tubes into centrifuge at 1000 RPM for 10 minutes.
4. During this time, clean out any trash in the hood. Place into the hood filter holders, a few 12 ml syringes, a clean hemocytometer, nitex cloth and a 100 ml bottle.
5. Clean off the stir bar that was in the other 100 ml bottle and place it back in the alcohol

TRITURATION

1. After 10 minutes take the centrifuge tubes back to the hood.
2. Using the vacuum suck off the liquid off the pellet in each tube.
3. Add 15 ml of medium each tube (for more than 8 embryos- add 20 ml to each tube- you may want to use more than two centrifuge tubes).
4. Using a sterile cotton-plugged pipet, triturate the mixtures in each tube until the pellet is well broken up.

FILTRATION

1. Place one of the pre-sterilized filter holders at the end of a 12 ml syringe. Put two sterile nitex filters in holder and close holder.
2. Place filter holder and syringe on the top of an open, clean, sterile 100 ml bottle.
3. Swirl tissue-cell mixture and pour into the syringe. Place plunger into syringe barrel and push the liquid through the barrel.

Possible Problems:

A. If using more than 3 embryos its possible that the syringe will get clogged and liquid will start coming out the side of the filter. If this occurs, switch syringe to a new filter and push mixture through the new filter.

B. If the filter does not fit correctly in the filter holder, some of the tissue may get through the filter. This can be detected by swirling the mixture and checking to see if there are any chunks of tissue floating around. If there are any chunks in the filtered suspension, it will need to be refiltered.

It is a good idea to keep several sterile filters on hand.

COUNT CELLS

1. Stir mixture to make sure cells are evenly suspended and than extract a small amount of fluid using a cotton-stuffed pipet
2. Place a drop of the suspension into each chamber of hemacytometer.
3. Using an inverted microscope, count the cells in five of the inner boxes.
4. Add the two numbers together and multiply by 2.5×10^4. This will give the number of cells per ml. The calculations are outlined on the sheet used to detail every muscle culture.
5. Given the number of cells/ml, calculate the necessary dilution to obtain the proper preplating cell concentration. Presently we dilute to a concentration of 1×10^6 cells/ml.

PREPLATING

1. The diluted suspension is then placed into 10 cm tissue culture dishes at 10 ml/dish. Spread the suspension out in each dish by using the tip of the pipet. Don't shake the tray or all the cells willsettle in the middle, of the dishes.
2. Place dishes into the 5% CO_2 incubator for 45 min. (at 37^oC).
3. Remove one of the stir bars from alcohol. Place into petri dish lid. Place forceps along edge.
4. Remove water from 35 mm dishes and 24-well plates. Return 35 mm dishes to incubator.
5. Add regular medium to the 24-well plates (approx. 0.5 ml/well). Return plates to the incubator.
6. Clean 2 hemacytometers and place in the hood. Also place a clean 100 ml bottle in hood.

POST PRE-PLATING

1. In the hood, each 10 cm dish is swirled. The liquid is drawn up into 10 ml pipet and placed into the 100 ml bottle with the dry magnetic stir bar. Remove additional stir bar from alcohol.
2. Throw away the large petri dishes.
3. Stir the suspension and place a drop into each chamber of each hemacytometer using a cotton-stuffed pipet.
4. Count the number of cells in two of the large boxes in each chamber as shown below.
5. Average the values obtained for each chamber, add together the four averages. Multiply the total by 2.5×10^3 as noted on the record page.
6. This will give the number of cells/ml. Follow the outline on the record page to obtain a dilution factor for each plating density

PLATE 35mm DISHES

1. Place stir bar into a clean, sterile 500 ml of a 100 ml bottle if only plating a few dishes.
2. Measure medium needed for proper dilution and pour into bottle. Pour measured cell suspension into the bottle.
3. Place bottle on the stir plate and set stirring speed at a moderate rate.
4. Using a 10 ml plastic pipet, distribute 1.5 ml of cell suspension into each 35 mm dish. Fill all the dishes on the tray. Then, very carefully carry the tray over to the incubator and place into the incubator. Be careful not to cause too much movement of the fluid in the dishes because it will cause uneven distribution of cells on the bottom of the dishes

CLEAN-UP

1. Put left over eggs down the disposal.
2. Clean the hemacytomecers and return them to alcohol bath.
3. Throw out filters and place filter holders into distilled water.
4. Wash off stir bars and leave to dry.
5. Rinse out all bottles.
6. Throw out trash in hood.
7. Clean hood with alcohol.
8. Clean off forceps and scissors.

CHICK EMBRYO EXTRACT

The extra eggs for the prep need to be ordered two weeks before you plan on doing the extract. So when there are 8 to 10 tubes remaining order 5 t0 7 dozen eggs, depending on fertility. The embryos should be 11 days old, not 12 days because they are too big to go through the syringe. If eggs are incubated on Friday the prep can be done on Tuesday.

1. Thoroughly wash eggs with 70% alcohol. Place large 10 cm petri dish in hood, place a 2 1/2 inch stir bar in alcohol bath, remove and place in petri dish lid.
2. Remove 1-2 dozen embryos and place them into petri dish.
3. When dish is full, wash embryos twice with pucks
4. Place embryos in 50 cc syringe from which the plunger has been sterilely removed. Push embryos through syringe into 500 ml sterile pyrex bottle.
5. Repeat above procedure until all the eggs have been used.
6. Determine the volume of squished embryos in 500 ml bottle, add equals amount of pucks
7. Add dry stir bar to mixture, place on stir plate and let spin at moderate speed for 1-1 1/2 hr at room temperature.
8. Distribute this mixture between 2-3 centrifuge tubes for the JA-14 head of the model J-21C
9. Centrifuge for 1 hr at 14,000 rpm, 2°C. Have Ralph show you the procedure for operating centrifuge.
10. Remove bottles from centrifuge taking care not to disturb pellet.

11. In the hood remove supernatant; aliquot 10 ml into 15 ml tubes. Remove as much as possible before it gets too cloudy or has junk floating in it.
12. Date centrifuge tubes and write CEE on each tube , place in ultra cold freezer.
13. Wash the large centrifuge tubes and return to D220.

ARA-C (100 X) Cytosin-1-B-arabino-furanoside, Cytosine arabinoside

Make a 10^{-3} M solution of Ara-C. (@ 0.014 gms into 50ml of DDH_20). Aliquot solution into 1.5 ml tubes (sterile) and place in freezer. Ara-C into medium; Ara-C solution is 100 X so add 1 ml Ara-C to every 100 ml of medium. Kept in Bob Marshall's freezer

TRYPSIN

Add 50 ml (not 100 ml as per bottle directions) Pucks to lyophilized trypsin 0.25%. Aliquot trypsin 2 ml to a test tube and place these into the freezer. The trypsin should be kept in the freezer because it will start to eat itself up. Write T on each tube, date the container. If making T from crystal dissolve 250 mg into 50 ml pucks.

HEAT INACTIVATED HORSE SERUM

Place two bottles of thawed horse serum into water bath which has been preheated to 56^oC. Let sit for 1/2 hour. Distribute horse serum in to 50 ml centrifuge tubes for use in making medium. Place in Bob Marshall's freezer.

AUTOCLAVE H_2O

Fill 500 ml pyrex bottles with glass DDH_2O (from Shooter's tissue culture room) to 1/2 inch below bottle neck. Place autoclave tape on cap and autoclave for 35 min liquid cycle, 2 min dry time (leave cap slightly loose and place in pyrex dish with 1/2 inch of water).

4-AP (4-AMINOPYRIDINE) blocks $K^{\pm}$ channels, increases twitching

To prepare 10 X stock solution:	50 mM
	measure out quantity 47 mg 4-AP
	Add 10 ml pucks
	pH to 7.2 with HC1 or NaCI
	Freeze in 250 lambda aliquots
To use in assay:	thaw frozen stock 50mM, pH 7.2
	Add to 2.25 ml pucks.
	Add 6 lambda to 300 lambda well
	Final concentration is 10^{-4}

100X GLUCOSE

Measure out 11.0 grams of glucose and bring it up to 100 ml with DDH_2O.
Sterile filter solution with 0.2 micron filter, pour into sterile 100 ml pyrex bottle.
Store solution in refridge.

COLLAGEN COATING

Collagen (Calf skin) comes from Calbiochem. (Cat. # 234112)
Dissolve 50 grams of collagen in 100 ml 1:1000 HOAC (HOAC stir O/N in cold), autoclave solution to assure sterility.

PLATES:
24 well- 5 lambda/well of collagen solution. Spread with coater (a pasteur pipet which has been flame sealed and "arc"ed) Rotate plate after each row has been coated, place immediately into incubator.
96 well plate-2 lambda/well, spread with eppendorf tip.

DISHES:
place one drop of collagen from pasteur pipet into each dish, spread collagen with large collagen coater. After coating a tray place in incubator.

GLASS COVER SLIPS in dishes:
use collagen that has not been autoclaved (when collagen has been autoclaved it turns to gelatin and will not stick to glass) therefore when collagen is made it should be done as sterilely as possible.

FILTERS

Cut filters out of nytex mesh using the bottom of the filter holder as a pattern. Place two filters into each filter holder. Washer should go in (top) first, then the filters screw filter shut. Filter is then placed into a sterilizer pouch, nose down. Seal with autoclave tape. Autoclave on gravity cycle Steam sterile 25-30 min. Dry 5 min.

7.5% SODIUM BICARBONATE SOLUTION ($NaHCO_3$)

500 ml soln. 37.5 gms $NaHCO_3$
DDH_2O up to 500 ml
Autoclave or sterile-filter

MEDIUM

450 ml MEM
10% Horse serum
10 ml CEE (stays in –80°C freezer)
5 ml Penicillin/Streptomycin
5 ml L-Glutamine (dissolve in hot water bath)
Store medium in refrigerator

10X PUCK'S w/o Mg or Ca

4.0 grams KCl
3.2 grams NaH_2PO_4
95.0 grams NaCl
10.8 grams Na_2HPO_4 or 20.6 grams Na_2HPO_4-$7H_2O$
Add DDH_2O (not sterile) up to 1000 ml
Check pH and osmolarity of 1x solution
pH 7.1 - 7.4
osmolarity 310 - 330 mOsm

The osmometer is in Bob Marshall's room. Once corrections have been made the 10x Pucks should be sterile filtered and poured into two 500 ml sterile pyrex bottles. This solution is kept at room temp. or else the salts will come out of solution. Check when using to make sure this has not occurred.

PUCKS WITH GLUCOSE

50 ml 10X pucks
5 ml glucose (100X)
Add sterile distilled water to 500 ml
Store in refrigerator