

ISOLATION AND GROWTH OF MOUSE PRIMARY MYOBLASTS

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Primary mouse myoblasts can be purified, stably transduced, and grown extensively in vitro. The transplantation of such cells can thus be used as a method of gene delivery. Primary cultures derived from skeletal muscle consist mainly of myoblasts and fibroblasts and the following protocol describes the purification of myogenic cells from such a mixed population. During the initial week or two of culturing, selective growth and passaging conditions allow the myoblasts to become the dominant cell type, and after two to three weeks in culture, nearly 100% of the cells stain positive for the myoblast specific protein desmin. Primary myoblasts can be isolated from mice of any age, but isolation from neonatal mice gives a greater yield of myogenic cells.

Materials

70% ethanol in a squirt bottle

Sterile phosphate buffered saline (PBS)

Collagenase/dispase/CaCl₂ solution

F-10-based primary myoblast growth medium

F-10/DMEM-based primary myoblast growth medium

Fusion medium

Neonatal mice, preferably 1-3 days old

Sharp curved surgical scissors

2 pairs of fine forceps

Low power stereo dissecting microscope

Sterile razor blade

80 μ m nylon mesh (e.g. Nitex; Tetko, Inc.; Monterey Park, CA)

Small sterile funnel

Collagen-coated tissue culture dishes: 35 mm, 60 mm, 100 mm, and 150 mm sizes

Humidified 37°C, 5% CO₂ incubator

Inverted microscope

Isolate limb muscle

1. Sacrifice 1-5 neonatal mice by decapitation or CO₂ inhalation.
2. Rinse the limbs with 70% ethanol and remove them with sterile scissors. Dissect the muscle away from the skin and bone with sterile forceps.

Dissection is easier if done under a stereo dissecting microscope. Store the muscle tissue in a culture dish on ice in a drop of sterile PBS as successive limbs are being processed, maintaining sterility in the accumulated tissue.

Dissociate muscle cells

3. Add enough PBS to keep the tissue moist, and mince to a slurry with razor blades in the culture dish.

Do this and all subsequent steps in a sterile tissue culture hood.

4. Add approximately 2 ml of collagenase/dispase/CaCl₂ solution per gram of tissue and continue mincing for several minutes.

For the amount of tissue obtained from 1-5 neonatal pups, 0.5 ml of the enzyme solution is usually sufficient.

5. Transfer the minced tissue to a sterile tube and incubate at 37°C until the mixture is a fine slurry, usually about 20 min. Several times during the incubation, gently triturate with a plastic pipette to break up clumps.
6. If desired, the slurry can be filtered through a piece of 80 µm nylon mesh in a sterile funnel to remove large pieces of tissue.
7. Centrifuge the cells for 5 min at 350 x g. Resuspend the pellet in 2-4 ml of F-10-based primary myoblast growth medium depending on the amount of tissue processed, and plate in a 35-60 mm collagen coated culture dish.

It is common to see a great deal of debris and very few recognizable cells; however, after two days, many cells will be evident and the debris will be rinsed away during the first change of medium.

Enrich for myoblasts

8. Incubate in a 37°C 5% CO₂ incubator and change the medium every 2 days, using F-10-based primary myoblast growth medium. Do not leave cells in the same culture dish for more than 5 days, regardless of cell density.

Primary myoblasts grow best when dense. Do not grow them at less than approximately 10% confluence, but also do not allow them to become too crowded, or else they may start to differentiate or die. Split them at no more than 1:5 dilution. The F-10-based primary myoblast growth medium gives myoblasts a growth advantage over fibroblasts.

9. When the cells are ready to be split, remove the cells from the dish using PBS with no trypsin or EDTA. This is accomplished by aspirating off the growth medium, rinsing the dish with PBS, leaving a small amount of PBS in the dish, and hitting the dish very firmly in a sideways fashion against the edge of a table top to dislodge the cells.

It can take several minutes of incubation in the PBS before the cells can be knocked off of the tissue culture plastic. However, the myoblasts will freely come off during this treatment and many fibroblasts will be left behind.

10. Preplate the cells for 15 min on a collagen coated dish before moving the cells remaining in suspension to a new collagen coated dish.

This tends to leave behind cells that stick quickly, which are predominantly fibroblasts. To avoid leaving myoblasts behind, swirl the liquid in the dish, tilt it towards the pipette, and jostle the dish from side to side while slowly removing the cell suspension. Failure to do this can result in a significant number of myoblasts trapped in the meniscus.

11. Repeat steps 9 and 10 for the initial week of culture expansion or until most of the fibroblasts are gone from the culture.

Fibroblasts tend to be very flat when grown on collagen, whereas myoblasts are more compact and much smaller in diameter. Myoblasts can also be identified by immunofluorescent staining for desmin.

12. After the fibroblasts are no longer visible in the culture, the medium can be changed to F-10/DMEM-based primary myoblast growth medium.

This medium allows myoblasts to grow faster and to higher densities. However, it also removes the selective growth advantage of myoblasts over fibroblasts, and thus should not be used until after the fibroblasts are gone. This medium can be changed every three days instead of every two, but care should still be taken to avoid overgrowth of cells. Primary myoblasts can be frozen for storage using standard cell culture protocols.

After approximately one week of growth, the myoblasts usually go through a crisis period during which a significant fraction of the population (sometimes the majority) dies. This tends to occur at the time that the fibroblasts have disappeared from the culture, although a direct correlation has not been confirmed in our laboratory. The remaining myoblasts soon repopulate and have a remarkable proliferative ability (up to 100 doublings) but still retain the ability to differentiate under the proper conditions.

Confirm ability to differentiate in culture

13. If desired, the potential of the myoblasts to differentiate in culture can be assessed by replacing the medium with fusion medium.

The medium must be changed daily. Start with an approximately 30-50% confluent dish of cells. Within several days to one week, large multinucleated myotubes should be quite obvious. After approximately one week in fusion medium, the myotubes can sometimes be observed to twitch randomly as the contractile machinery assembles.

REAGENTS AND SOLUTIONS

Collagen-coated tissue culture dishes

Add 1 ml concentrated acetic acid to 179 ml H₂O

Sterile filter through 0.2 μ m filtration unit

Add 20 ml 0.1% calf skin collagen in 0.1 N acetic acid (Sigma)

Incubate in plastic tissue culture dishes overnight at room temperature

Remove collagen solution (can be reused; store at 4°C)
Rinse dishes well with sterile H₂O; allow to dry
Store at room temperature for at least 6 months

Collagenase/disperse/CaCl₂ solution

1.5 U/ml collagenase D (Boehringer Mannheim Corp.) from -20°C frozen aliquot
2.4 U/ml disperse II (Boehringer Mannheim Corp.) from -20°C frozen aliquot
2.5 mM CaCl₂
Use immediately.

F-10-based primary myoblast growth medium

400 ml Ham's F-10 nutrient mixture (GIBCO BRL)
100 ml fetal calf serum (HyClone Laboratories, Inc.) (20% final)
50 µl 25 µg/ml basic fibroblast growth factor (human; Promega Corp.) in 0.5% bovine serum albumin/PBS; freeze aliquots only once at -20°C (2.5 ng/ml final)
5 ml 100x penicillin/streptomycin (GIBCO BRL; final concentration is 100 U/ml and 100 µg/ml, respectively)
Store at 4°C for at least 1 month

F-10/DMEM-based primary myoblast growth medium

Same as above but use 200 ml F-10 and 200 ml Dulbecco's Modified Eagle Medium (DMEM; GIBCO BRL) in place of the 400 ml F-10

Fusion medium

95 ml DMEM
5 ml horse serum (HyClone) (5% final)
1 ml 100x penicillin/streptomycin solution (above)
Store at 4°C for at least 1 month

Phosphate buffered saline (PBS), 10x stock

2.0 g KCl
2.0 g KH₂PO₄
80.0 g NaCl
11.4 g Na₂HPO₄ (anhydrous)
Dissolve Na₂HPO₄ in 200 ml ddH₂O with stirring and heating
Dissolve remaining three salts in 700 ml ddH₂O; combine both solutions
Adjust pH to 7.4 with 5N NaOH
Adjust volume to 1 liter and autoclave or filter. Store at room temperature.