

Human Muscle Cell Isolation – Updated February 2011

Reagents/Tools/Equipment:

- Biopsy transfer medium (ice-cold; use within 1 week)
 - 20% fetal bovine serum, characterized (FBS, Hyclone SH30071.03)
 - 2% chick embryo extract (CEE, protocol below)
 - 2.5 ng/mL basic fibroblast growth factor (Millipore GF003-AF)
 - 1% antibiotics and antimycotics (Cellgro 30-004-CI)
 - in Ham's F10 medium (Cellgro 10-070-CV)
- Hanks Balanced Salt Solution (HBSS; Gibco 14175)
- collagenase/dispase solution (need 5 mL per 0.5 g biopsy - make fresh):
 - 1 mg/mL collagenase IV (Worthington LS004186; store 100 mg/ml stock at -20°C)
 - 2.5 mM CaCl₂ (EMD 3000-OP; store 250 mM stock, filter-sterilized, at 4°C)
 - 2.4 U/mL neutral protease/dispase (Worthington LS02104; store 50 U/mL stock at -20°C)
 - in HBSS
- HMP growth medium (store at 4°C for up to 1 week)
 - 20% FBS
 - 0.5% CEE
 - 1% antibiotics and antimycotics
 - 1.2 mM CaCl₂
 - in Ham's F10 medium
- 2X freeze medium (make fresh; use ice-cold)
 - 50% FBS
 - 20% DMSO (Sigma D2650)
 - 30% HMP medium
- TrypLE Express (Gibco 12605-028)
- phosphate buffered saline w/o Ca²⁺ or Mg²⁺ (PBS, Cellgro 21-040-CM)
- fine forceps, sterilized
- fine iris scissors or spring scissors (curved), sterilized
- 70% ethanol
- 10 cm petri dishes
- 6 cm tissue culture dishes
- 6 & 10 cm tissue culture dishes, coated with 0.1% gelatin
- 5 mL, 10 mL plastic serological pipettes
- automatic pipettor
- 100 um & 40 um cell strainers (BD Falcon 352360/352340)
- 50 mL tubes (BD Falcon 352098: these fit the cell strainers)
- 15 mL tubes
- hemocytometer
- dissecting microscope; inverted tissue culture microscope
- class II biosafety cabinet
- 37°C water bath
- 37°C CO₂ incubator (5% CO₂)
- clinical centrifuge with biohazard containment

Protocol: Day 1

1. Approximately 500 mg skeletal muscle biopsy material will be dissected into $<5 \text{ mm}^3$ fragments, stored in ice-cold transfer medium (5-10 mL/biopsy), and shipped overnight on ice packs from the Kennedy Krieger Institute (Baltimore, MD) to Boston Biomedical Research Institute (Watertown, MA). Upon arrival, examine all tubes immediately for signs of leakage, freezing/crystallization, warming, etc. After assessing and recording condition, transfer tubes to ice.

2. Prep: Sterilize instruments, dissecting scope and all surfaces with 70% ethanol; prewarm collagenase/dispase solution and growth medium to 37° C. For each biopsy, you will need one empty 10 cm petri dish, two 10 cm petri dishes with HBSS, and one empty 6 cm tissue culture dish, labelled appropriately.

3. Clean tissue: Sterilize the first sample tube with 70% ethanol and transfer it to the biosafety cabinet. Photograph biopsy in original container, and then transfer contents (medium and tissue fragments) into a labelled 10 cm petri dish - photograph again with the original container in the frame for documentation. Using sterile forceps, transfer tissue fragments to one of the 10 cm dishes with HBSS. Under the dissecting microscope, remove visible extraneous connective tissue (vessels, tendons, fat, etc) from muscle fragments, transferring the cleaned fragments to the second 10 cm dish with HBSS. When finished, transfer tissue fragments to one edge of a 6 cm tissue culture plate; if necessary, keep tissues wet with ~100 uL HBSS. (N.B. for samples that are excessively fibrotic and/or fatty, it may be impossible to clean tissue efficiently or at all; under these circumstances, proceed directly to step 4.)

4. Mince & digest tissue: In the biosafety cabinet, use sterile fine iris scissors to mince the wet tissue for 3-5 minutes. Add 3 mL of collagenase-dispase solution and mince for 2 more minutes. Add final 2 mL of collagenase-dispase solution; transfer dish to 37° C incubator for 15 minutes.

5. Trituration: After 15 minutes in collagenase-dispase solution, triturate tissue by pipetting up and down with a 5 mL plastic serological pipette 15 to 20 times, and return to incubator. Repeat incubation/trituration two more times (i.e. total of 45 minutes incubation, triturating after every 15 minutes). Using a 20X phase objective, verify that muscle fibres are dissociated and that single cells are not clinging to the fibres. If bundles of myofibres are still observed after 45 minutes, incubate for an additional 15 minutes and re-triturate. (N.B. Usually tissue is dissociated within 45 minutes-60 minutes, but cultures have been successfully established following up to 90 minutes in enzyme).

6. Filtering: When tissue dissociation is complete, filter contents through a 100 um cell sieve placed on top of a 50 mL tube. Use 3 mL of HBSS to rinse the plate and cell sieve. Filter next through a 40 um cell sieve. Following filtration, transfer cells to a 15 mL tube and centrifuge for 5 minutes at 1000xg at room temperature.

7. Counting and plating: Resuspend the cell pellet in 5 mL of HMP growth medium and count cells. Plate cells on a 6 cm dish coated with 0.1% gelatin. Incubate at 37° C/5% CO₂.

8. Repeat steps 2 through 9 for each biopsy.

9. **Day 2:** Examine all cell cultures – monitor cultures for signs of cell attachment, clumping, contamination, etc.

10. **Day 3 and onwards:** Feed cells with fresh HMP medium daily.

11. **Passaging & Freezing:** When cells are ~50-70% confluent, aspirate medium, rinse with PBS and incubate with TrypLE Express (Invitrogen) for 5 minutes at 37° C or until cells have detached. Neutralize TrypLE with growth medium and count cells. At this first passage, a rough population doubling number is assigned (equal to the number of days in culture; subsequent PD additions will be based on cell counts). To expand cells, seed at ~1,000 to 2,000 cells/cm² on a gelatin coated dish and feed HMP daily. To freeze cells, adjust volume accordingly with HMP and add an equal volume of ice cold, 2X freeze medium, and mix well by pipetting. Freeze cell aliquots in cryovials in the liquid nitrogen vapour phase for at least 1 hour before transferring to liquid nitrogen or -140° C freezer.

Additional protocols:

0.1% gelatin-coated plates: dissolve 1 g gelatin (Sigma G9391) in 1 L tissue-culture grade water; mix well and autoclave; store at room temperature. To coat dishes, add sufficient volume of 0.1% gelatin to cover surface, aspirate, and air-dry in biosafety cabinet. Store at room temperature.

Chick embryo extract: (Adapted from F. Stockdale's lab protocol.) Harvest Day 12 embryos from 5 to 10 dozen fertilized White Leghorn chicken eggs (Charles River, SPF Premium Eggs), using sharp-point scissors to pierce and then cut the top off of the eggshell. Decapitate as close to the head as possible with sterile scissors, and rinse embryo bodies 2X in ice-cold HBSS. Macerate chick embryos by pushing them through a 60 ml syringe, and collect in a chilled, sterile graduated cylinder. Add an equal volume of ice-cold HBSS, transfer to a sterile beaker and stir, covered, for one hour at 4°C. Centrifuge at 10,000 rpm for 1 hour at 4°C and collect supernatant. (Optional: filter through 0.45 um filter system; this can be difficult depending on how clean the supernatant is, so we tend to skip this step and add an additional centrifugation step as described below.) Store aliquots in 15 mL tubes at -20°C, or -80°C for long-term storage. To use, place 15 mL aliquot in a room temperature water bath just until thawed, then spin for 30 minutes at 4,000 rpm at 4°C to pellet any remaining contaminants from the processing. Add supernatant to medium and filter through 0.2 um filter system; aliquot leftover CEE and freeze.