Protocol for the isolation of a heterogenous muscle-derived cell population, Goodell Lab style!

Shannon L.McKinney, KathyJo Jackson, Tiejuan Mi, Susan Majka, Margaret A. Goodell

This protocol is employed for the purification of a population of muscle-derived cells from skeletal muscle of C57Bl/6 animals. The resulting preparation is a mixture of many cell types including satellite cells (a.k.a., muscle progenitor cells) and hematopoietically active muscle-derived cells. This protocol is based on that described by Yablonka-Reuveni *et al*., with slight modifications[1].

1. **Excise the muscle from the lower limbs of 6-8 week-old mice and place into a 10 cm tissue culture dish containing approximately 10 mL of Hanks Balanced Salt Solution (HBSS+, Gibco).**

   Notes: The use of younger animals will result in a higher yield.

   The use of female animals will also result in a higher yield relative to male animals. This yield difference is most likely due to the fact that female muscle is less fibrous and therefore more easily minced and digested than male muscle.

   If your goal is to purify satellite cells, than you may also excise the diaphragm at this step; the diaphragm is enriched for satellite cells relative to skeletal muscle.

2. **Remove any bones and tendons from the muscle.**

3. **In a minimal amount of HBSS+, thoroughly mince the muscle into a fine slurry of 1mm^2 or smaller particles.**

   Notes: It is critical that the muscle be thoroughly minced: Extensive mincing gives the collagenase a larger surface area to "attack" and facilitates the digestion. Therefore, though your hands may tire, it is important to spend a decent amount of time on this step and do a thorough job. Just think, you'll have the strongest fingers in the lab and be a competitive thumb wrestler! Though you should use a minimal amount of media for this step, be sure that the tissue does not dry out. We recommend you use one pair of curved mincing scissors, two pairs of forceps (one large and one small), and a pair of sharp surgical scissors (see end of protocol for recommended vendors and catalog numbers).

4. **Transfer the minced muscle to a 50 mL conical tube and spin down at 2000 rpm for 3 minutes in a clinical centrifuge.**

   Notes: Transfer only two animals worth of minced muscle per 50 mL conical tube. This amount of muscle seems to work best for the Percoll™ spin step later in the protocol.

5. **Discard supernatant. Add an equivalent volume of 0.2% type II collagenase (Worthington Biochemicals) to the tube (usually 4-5 ML). Mix well and incubate mixture in a circulating water bath for 30 minutes.**

   Notes: Some collagenases are more equal than others! Collagenase type II from Worthington Biochemicals definitely results in the best yields. Do not use a different type or a different company's. Changing the type of collagenase will have a large negative impact on your yield.
   A thorough collagenase digestion is key to a good yield. Therefore the suspension should be mixed well every ten minutes while it is incubating. Furthermore, if after 30 minutes, you still note large pieces of undigested muscle, the digestion should be extended another 15 minutes. This step should result in a very liquidy suspension in which very few large muscle pieces are apparent.

   Use only FRESHLY prepared collagenase. Once the collagenase has been suspended in HBSS+, it does not last more than two days at 4ºC. The use of older resuspended collagenase will result in lower yields.

6. **(This step is optional) Add approximately 15 ML of HBSS+ to the digestion suspension. Spin down at 3000 rpm for 5 min. Discard supernatant. Add an equivalent volume (3-4 ML) of 0.25% trypsin (Gibco). Incubate in a circulating water bath at 37ºC for 30 minutes, vortexing every 10 minutes. Proceed to step 7 (do not spin down digest).**

   Caveat: This step will result in slightly higher yields but will also have a detrimental effect on your ultimate cell viability. If you are planning to stain your muscle-derived cells with Hoechst Dye, or if you are concerned about cell viability, it is recommended that this step is skipped.

7. **If you have skipped the trypsin digestion, fill the 50 ML conical containing the collagenase digest to the brim with HBSS++ to wash away collagenase. Spin at 3000 rpm for 5 minutes. Discard supernatant. Add approximately 10 ML of warm DMEM/10% Horse Serum/1% glutamine/1% antibiotic (DMEM/HS) to digested muscle. Triturate sample 5x with 10 mL of DMEM/HS using a ten mL plastic pipet: this is done by drawing the mixture up and down inside the pipet repetedly in order to break up any remaining pieces of muscle tissue and release muscle progenitors from beneath the basal lamina of the intact fibers. After each trituration, transfer medium to fresh 50 mL conical tube. Avoid transferring tendons.**

8. **Pass transferred triturate through a 100µm filter (Falcon, cell stainer, #352360) into a fresh 50 mL conical. Collect cells by centrifugation at 3000 rpm for 5 minutes and resuspend in 3 ML HBSS+.**

9. **Prepare Percoll™ (Amersham Pharmacia Biotech AB, Uppsala, Sweden) gradient: First, make a 90% Percoll™ solution by diluting 9 parts Percoll™ with one part 10x PBS (GIBCO). Next, prepare 40% and 70% Percoll™ solutions by**

diluting the 90% Percoll™ solution with 1x PBS (GIBCO) to the appropriate concentration. You can prepare the 90% Percoll™ solution ahead of time, but the 70% and 40% solutions should be made fresh. You will gently overlay 3 mLs of 70% Percoll™ with 3 mLs 40% Percoll™ in a 15 mL conical tube.

10. **Gently overlay cell suspension onto Percoll™ gradient. Wash 50 mL tube that cells were transferred from with 3 ML HBSS+ and overlay this onto same Percoll™ gradient. Centrifuge at 2500 rpm for 20 minutes with the break off at 25°C.**

    Notes: Do not overlay more than two animals worth of muscle-derived cell suspension per Percoll gradient.

11. **Remove cells from 70% Percoll™/40% Percoll™ interface and transfer to fresh 50 mL conical. Fill conical to brim with HBSS+ in order to wash away Percoll™ and collect cells via centrifugation.**

12. **Count cells via hemocytometer. Typical cell yields range from $1x10^6$ to $2x10^6$ cells/mouse.**

References:

1) Yablonka-Reuveni, Z., and Nameroff, M. (1987). Skeletal muscle cell populations. Separation and partial characterization of fibroblast-like cells from embryonic tissue using density centrifugation. Histochemistry *87*, 27-38.

<u>Solutions and Dissection tools</u>

HBSS+: Hanks balanced salt solution (GIBCO) with 1% glutamine and 1% antibiotic.

DMEM/HS: DMEM high glucose (GIBCO), 10% horse serum, 1% glutamine, 1% Antibiotic

Dissecting forceps heavy, rounder points with corrugated tips: VWR, 25720-043
Dissecting forceps, fine point: VWR, 25715-043
Straight delicate dissecting scissors, 5”: VWR, 25608-360
Castro-Viejo scissors, short, delicate spring handle, blade slightly curved: VWR, 25608-586

Brief outline of protocol:

1. Remove muscle from lower limbs and diaphram of animals and transfer to 10 cm tissue culture dish containing HBSS+.
2. Remove bones and tendoms from muscle.
3. Thouroughly mince muscle in a minimal amount of HBSS+.
4. Transfer minced muscle to fresh 50 mL conical tube and centrifuge @ 2000 rpm for 3 minutes.
5. Discard supernatent. Add an euqivalent volume of 0.2% collagenase type II. Mix well and incubate in a 37ºC water bath for 30 minutes, mixing well every 10 minutes.
6. (optional) Add 15 mL of Hanks+ to the tube. Spin at 3000 rpm for 5 minutes. Discard supernatent. Add an equivalent volume of 0.25% trypsin. Incubate at 37ºC for 30 minutes in a water bath for 30 minutes, mixing well every 10 minutes.
7. If you skipped step 6, fill the conical tube with Hanks+ to wash away collagenase and spin at 3000 rpm for 5 minutes. Add approximately 10 mL of DMEM/HS to tube. If you proceeded with step 6, add the 10 mL of DMEM/HS directly to the tube containing the trypsin and proceed with next step.
8. Triturate the sample with 5 aliquots of DMEM/HS. Transfer clear titurate to fresh 50 mL conical tube.
9. Pass transferred triturate through a 100 μm filter. Collect cells by centrifugation at 3000 rpm for 5 minutes and resuspend in 3 mL of Hanks+.
10. Prepare Percoll gradient.
11. Gently overlay cell suspension onto Percoll gradient. Wash 50 mL tube with 3 mL of Hanks+ and add that overlay onto same gradient in order to collect maximum number of cells.
12. Centrifuge at 2500 rpm for 20 minutes with the break off at 25°C.
13. Remove cells from 70%/40% Percoll interface and transfer to fresh conical tube. Fill conical to brim with 1x PBS in order to wash out Percoll . Collect cells by centrifugation.
13. Count cells via hemacytometer. Typical cell yields range from $1x10^6$ to $2x10^6$ cells/mouse.