

Video Article

Purification of Progenitors from Skeletal Muscle

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Abstract

Skeletal muscle contains multiple progenitor populations of distinct embryonic origins and developmental potential. Myogenic progenitors, usually residing in a "satellite cell position" between the myofiber plasma membrane and the laminin-rich basement membrane that ensheathes it, are self-renewing cells that are solely committed to the myogenic lineage^{1,2}. We have recently described a second class of vessel associated progenitors that can generate myofibroblasts and white adipocytes, which responds to damage by efficiently entering proliferation and provides trophic support to myogenic cells during tissue regeneration^{3,4}. One of the most trusted assays to determine the developmental and regenerative potential of a given cell population relies on their isolation and transplantation⁵⁻⁷. To this end we have optimized protocols for their purification by flow cytometry from enzymatically dissociated muscle, which we will outline in this article. The populations obtained using this method will contain either myogenic or fibro/adipogenic colony forming cells: no other cell types are capable of expanding *in vitro* or surviving *in vivo* delivery. However, when these populations are used immediately after the sort for molecular analysis (e.g qRT-PCR) one must keep in mind that the freshly sorted subsets may contain other contaminant cells that lack the ability of forming colonies or engrafting recipients.

Video Link

The video component of this article can be found at <https://www.jove.com/video/2476/>

Protocol

1. Tissue Collection:

1. Use mice between 7 and 12 weeks old. Usually, at least two animals are used: one is to set up the FACS single-color controls required for compensation and for fluorescence-minus-one isotype control samples. The other is the actual sample for sorting.
2. Euthanize the mice by CO₂ inhalation or according to an ethics protocol of choice. Place mice on a paper towel, facing up, and spray with 70% ethanol thoroughly to prevent contamination with the mouse fur.
3. Lift the skin in the abdominal region with forceps, then make a small longitudinal cut with sharp scissors.
4. Peel the two flaps of skin in opposite directions (up and down) to completely expose the hind limb muscles underneath.
5. Excise the muscle tissue by inserting and extending the scissors along both sides of the tibia.
6. Repeat the same process for the femur to extract the muscle tissue.
7. Place all excised muscle tissue in 60mm petri-dishes, using one dish for the tissue from each mouse.
8. Repeat the above for all mice.

2. Dissociate Tissue Into Single Cells by Enzymatic Digestion:

Throughout this section of the protocol, use sterile reagents and tools and work under sterile conditions.

9. Add 2 mL of Collagenase II (Sigma, cat#6885, 500u/ mL) and 20 µL of 250mM CaCl₂ stock to each Petri-dish.
10. Tear the muscle tissue into small pieces (about 1 mm³) with two forceps (one serrated, one with 2 teeth, from Fine Science Tools, cat#11051-10, 11154-10), paying attention to remove and discard as much contaminated non-muscle tissue as possible.
11. Cover the Petri-dishes and incubate them at 37 degrees Celsius for 30 minutes.
12. Retrieve the Petri-dishes and use a 3 mL syringe plunger to thoroughly mash the tissue pieces. Use one plunger for each sample to avoid contaminations.
13. Add some (~ 5 mL) sterile cold PBS to each Petri-dish and transfer the tissue sludge into a 50mL Falcon tube (rinse the Petri-dish if necessary to recover all the tissue)
14. Shake the Falcon tube vigorously, fill the tube with PBS, centrifuge @800rpm x 5 minutes (Eppendorf bench top Model :5810R), and aspirate the supernatant (repeat 3 times)
15. Add 1mL mixture of Collagenase D (Roche, Cat# 11088882001, 1.5u/ mL) /Dispase II (Roche, Cat# :04942078001, 2.4u/ mL) and 10µL of CaCl₂ (250mM) to each tube, incubate at 37 degrees Celsius with rotation for one hour.
16. Add 5-10 mL of cold, sterile PBS, vortex and pipette thoroughly to homogenize the tissue.

17. Fill up tubes with PBS and mix well.
18. Transfer the liquid to a 40µm cell strainer placed on top of a 50 mL Falcon tube to filter out the remaining large pieces of tissue. Divide each sample evenly into three 50mL Falcon tubes. Fill up each of the tubes with PBS then centrifuge @1600rpm for 5 minutes at 8 degrees Celsius.
19. Discard the supernatant and collect cells for staining.

3. Antibody Staining and Sorting:

Throughout this section of the protocol, use sterile FACS and collection buffers and work under sterile conditions. Commercial antibodies are generally considered sterile if properly handled.

20. For single color and isotype control samples, resuspend the sample in 1mL FACS buffer and divide the suspended cells in nine 1.5mL pre-labelled Eppendorf tubes (100 µL each tube).

1. Set up single color staining mixes according to the following table:

ATTENTION: The final concentration of isotype control antibodies in the stain should match that of the primary antibody they are the control for. For example, if anti-Sca-1 is used for 1 µg/10⁶ cells, its corresponding isotype must be used at the same concentration.

Tube #	Name of tube	Company	Cat#	clone	isotype	dilution
1	Non-stain					
2	Hoechst	sigma	B2261			2-5ug/ml
3	PI	sigma	P4864			1ug/ml
4	CD31-FITC CD45-FITC	ebioscience	11-0311-85	390	Rat IgG2a	500
		House made		I3/2	Rat IgG2b	500
5	Sca1-PECY7	ebioscience	25-5981-82	D7	Rat IgG2a	5000
6	α-7 APC	House made		R2F2	Rat IgG2b	1000

2. Set up isotype control staining mixes using the following table:

Tube #	Name of tube	Hoechst	PI	CD31-FITC	CD45-FITC	Scal-PE-CY7	α-7-APC	Rat-IgG2b-APC	Rat-IgG2a-pecy7	Rat-IgG2a-FITC	Rat-IgG2b-FITC
7	Iso-CD31 / CD45	+	+	-	-	+	+	-	-	+	+
8	Iso-Scal-pecy7	+	+	+	+	-	+	-	+	-	-
9	Iso-α-7-APC	+	+	+	+	+	-	+	-	-	-

3. Pipet the single color and isotope control staining mixes (we usually adjust the concentration of antibodies so that between 5 and 10 µL of staining mix are added to each sample) into the appropriate Eppendorf tube from Step 20. Mix well and incubate on ice for 1 hour.
21. Stain cells from sample mouse with 800µL of the antibody cocktail (below) for FACS cell sorting. Mix well and incubate on ice for 1 hour.
Antibody cocktail: Hoechst, PI, CD31-FITC, CD45-FITC, Sca1-PECY7, α-7 APC
ATTENTION: The optimal ratio of antibodies to cells must be determined by individually titrating each antibody, as significant differences may exist between batches. The optimal amount of antibody to be used should be expressed in µg of antibody per 10⁶ target cells and kept constant when using the same batch of antibody. Clearly, if the number of target cells does not change significantly between experiments, or if the staining volume is scaled with the number of cells, a fixed dilution of each antibody can also be used.
22. Wash: Add approximately 1mL of FACS buffer to each of the 9 control tubes; add approximately 20mL of FACS buffer to the sample tube.
23. Centrifuge the Eppendorf tubes @ 3000rpm for 5 minutes (Eppendorf bench top centrifuge, Model:5417C). Centrifuge the sample tube @1600rpm for 5 minutes (Eppendorf bench top centrifuge, Model:5810R).
24. Aspirate and discard the supernatant from all the tubes.

25. Resuspend cells in the FACS buffer (1mL for each of the controls, 4mL for the sorting sample), Pipette cells into "FACS tubes" through the cell strainer cap (BD Falcon cat# 352235, 40µm pore size) to remove remaining clumps that may affect the cytometer.
26. Set up the FACS Sorter with single color controls and isotype controls.
27. Collect sorted cells according to the following table in a 3.5 mL collection buffer (95% DMEM, 5%FBS) separately. Normally, when harvesting both hind limbs, a single wild type mouse could yield about 150,000 myogenic progenitor cells or 100,000 adipogenic progenitor cells, with the viability above 95%, as judged by reanalyzing sorted cells by FACS at the end of the procedure (Figure 1)

Definition of myogenic and adipogenic progenitors based on surface staining

Antibody/stain	Myogenic progenitor (MP)	Adipogenic progenitor (AP)
Hoechst	+	+
PI	-	-
CD31-FITC	-	-
CD45-FITC	-	-
Sca1-PECY7	-	+
α-7 APC	+	-

4. Transplantation:

28. Centrifuge sorted cells @1500rpm for 5 minutes, remove the supernatant and transfer cells to autoclaved Eppendorf tubes (1.5 mL), rinse cells with 500 µL of sterile PBS (serum free!), then centrifuge @ 3000rpm for 5 minutes. Maintaining all reagents in sterile conditions and working in a tissue culture hood, resuspend myogenic progenitors in PBS, or adipogenic progenitor in Matrigel (BD cat# 354234) at approximately 10⁶ cells /mL.
29. Transplant myogenic progenitors or adipogenic progenitor to recipient mice.
 1. Anaesthetize the mouse with isoflouran according to your institution policy.
 2. Shave the hair around the injection region, then sterilize the skin by rubbing with 70% ethanol
 3. Inject 20µL of myogenic progenitors or adipogenic progenitors (= 20k cells) using a 3/10 CC insulin syringe (BD cat# 309300).
30. After an appropriate time (three weeks works well, but myofibers expressing donor-derived transgenic markers are usually evident within one week after transplant), anaesthetize the recipient mice from step 29 by intraperitoneal injection of 400 µL Avertin (Sigma Cat# T04840-2, 25 mg/ mL), then perfuse transcardially with 50mL PBS (contains 10 µM of EDTA) first, followed by 15mL of 4% PFA. Collect target tissue, post-fix with 4% PFA overnight if required, then transfer to 20% sucrose overnight. Embed in OCT, freeze and cryosection.

5. Representative Results:

When MP are transplanted into mouse skeletal muscle, they readily fuse with pre-existing myofibers. Therefore any genetic label present in the donor cells will be easily detectable in the fibers that received them. Figure 2 shows an example where the transplanted cells expressed human alkaline phosphatase, revealed histochemically.

When AP are transplanted the result is highly dependent on the environment of the transplantation site: when transplanted subcutaneously these cells give origins to adipocytes and myofibroblasts (see figure 2). In many other sites, including muscle, they do not survive unless fatty degeneration has been induced³.

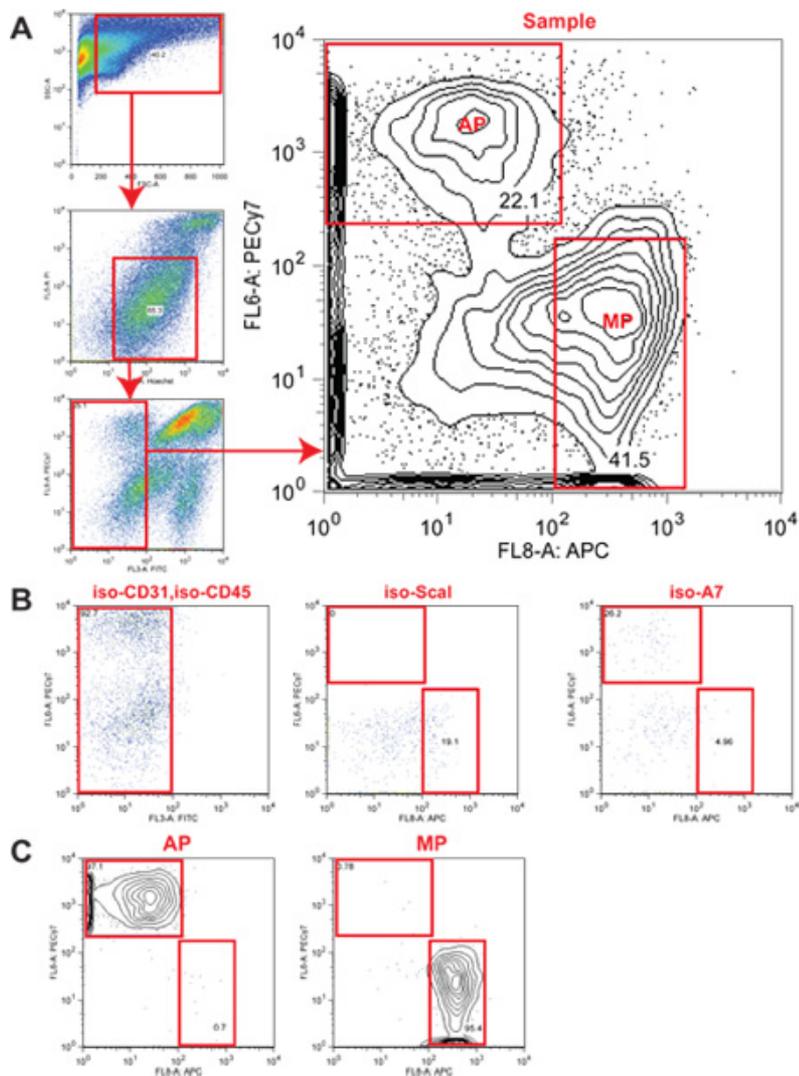


Figure 1. Sorting strategy for the isolation of progenitor populations from skeletal muscle. (A) Sorting strategy: Viable cells were identified based on forward scatter and side scatter. Hoechst staining was used to exclude nuclear debris and propidium iodide (PI) staining to exclude dead cells. Hematopoietic (CD45) and endothelial (CD31) cells were excluded from the sorting gates. The $\alpha 7^+$ Hoechst⁺ PI⁻ CD45⁻ CD31⁻ Scal⁻ subset contains all myogenic progenitors (MP). The Scal⁺ Hoechst⁺ PI⁻ CD45⁻ CD31⁻ $\alpha 7^+$ population contains adipogenic progenitors (AP). (B) Fluorescence-minus-one (FMO) isotype controls confirm the specificity of the stain. (C) Purity checks of MP and AP subsets after sorting.

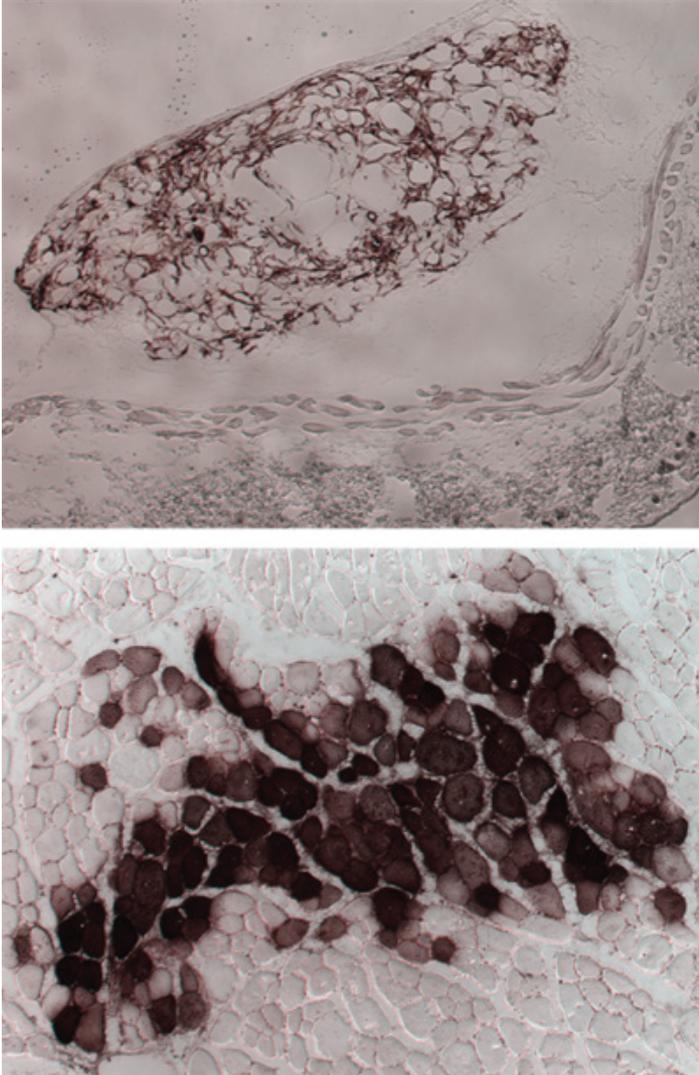


Figure 2. Representative results following MP and AP transplantation. AP were transplanted subcutaneously (top panel) and MP intramuscularly (bottom panel). In both cases, donor cells originated from a mouse expressing transgenic human alkaline phosphatase, identified by the brown staining.

Discussion

The goal of this protocol is to strike a reasonable balance between high yields and high viability of the purified cells. The most critical step in ensuring that healthy cells are recovered is, predictably, the enzymatic dissociation of the starting tissue. The handling of the tissue should be particularly gentle, which is hard to demonstrate or appreciate even in an audiovisual format. Another key factor is the length of the procedure. The longer it takes to go from the donor to the recipient animal, the lower the viability and therefore, the engraftment efficiency. Should there be any question about viability that is not immediately answered by looking at the frequency of PI positive event in the purified cell samples after sorting, we suggest that a limiting dilution assay to measure clonogenicity is performed³. Typical sorts from undamaged muscle routinely yield about 1 in 15-20 cells capable of initiating myogenic or fibro/adipogenic colonies *in vitro*. While essentially 100% of the colonies initiated from MPs contain differentiated myotubes, only about one third of colonies obtained from FAPs contain adipocytes in addition to smooth muscle actin positive myofibroblasts.

Disclosures

No conflicts of interest declared.

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