

Chapter 2

Isolation of Muscle Stem Cells from Mouse Skeletal Muscle

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Abstract

Isolation of muscle stem cells from skeletal muscle is a critical step for the study of skeletal myogenesis and regeneration. Although stem cell isolation has been performed for decades, the emergence of flow cytometry with defined cell surface markers, or transgenic mouse models, has allowed the efficient isolation of highly enriched stem cell populations. Here, we describe the isolation of mouse muscle stem cells using two different combinations of enzyme treatments allowing the release of mononucleated muscle stem cells from their niche. Mouse muscle stem cells can be further isolated as a highly enriched population by flow cytometry using fluorescent reporters or cell surface markers. We will present advantages and drawbacks of these different approaches.

Key words Satellite cells, Muscle stem cell isolation, Enzymatic dissociation, FACS

Abbreviations

| | |
|------|------------------------------------|
| FACS | Fluorescent-activated cell sorting |
| TA | Tibialis anterior |
| GFP | Green fluorescent protein |
| FSC | Forward scatter |
| SSC | Side scatter |
| C/T | Collagenase D/Trypsin |
| C/D | Collagenase A/Dispase II |
| FBS | Fetal bovine serum |
| CD | Cluster of differentiation |

1 Introduction

Biochemistry and molecular or cell biology studies on specific organs require the isolation of highly purified cell types to assess stem cell properties and their role in growth and regeneration.

However, such isolation of mononucleated cells from solid tissues and organs requires enzymatic treatments that ultimately result in the destruction of the stem cell niche and the differential stripping of cell surface molecules. One example is skeletal muscle satellite (stem) cells that lie on myofibers, located between the sarcolemma and its surrounding basement membrane [1]. Most satellite cells are quiescent during homeostasis. Following intense exercise or muscle injuries, satellite cells activate, proliferate, and differentiate to renew damaged myofibers [2, 3]. Molecular markers are used to distinguish quiescent satellite cells from their progeny [4, 5]. Furthermore, several studies have suggested that quiescent satellite cells constitute a heterogeneous cell population [6–8]. To date, the transcription factor Pax7 is the most reliable marker known that identifies all quiescent satellite cells [9]. *Pax7* expression marks the upstream myogenic population from mid-embryogenesis to adulthood. Its expression persists in activated and cycling satellite cells, but it is downregulated during myogenic commitment and differentiation, as the differentiation transcription factor myogenin is upregulated. Therefore, the *Pax7* locus has been a target of choice to generate knock-in and transgenic animals, to introduce GFP reporter or Cre recombinase genes that permit the prospective isolation and characterization of satellite cells.

Several genetically modified mice with a GFP reporter, which recapitulate *Pax7* expression, have been generated, for example, *Tg:Pax7-nGFP* [10, 11] and *Pax7-ZsGreen* [12]. More recently, the generation of four tamoxifen-inducible *Pax7-CreERT2* mouse lines [13–16] has also opened the possibility to label most satellite cells with mouse reporter lines such as *R26^{mT/mG}* or *R26^{eYFP}*. In these situations, the specificity and efficiency of the *Cre* mouse lines and the efficiency of the reporter line need to be closely examined. These four lines have different characteristics; the *Tg:Pax7-CreERT2* is a transgenic that does not affect the endogenous *Pax7* locus [15]. The knock-in *Pax7^{CreERT2}* has the *Cre* sequence inserted in the 3'UTR of the *Pax7* locus [16], similar in strategy but distinct from another knock-in [13]. These knock-ins result in *Pax7* expression due to the IRES sequence. Finally, the *Pax7^{Cre}* is a knock-in/knockout, where the *Cre* gene is inserted in the first exon of the *Pax7* locus resulting in a null allele [14].

Other mouse models have been generated, but they do not mark the entire satellite cell population. While *Pax3* is expressed in all body (except the head) myogenic cells during development, in the adult, its expression, reported with the *Pax3^{GFP}* mouse, is restricted essentially to some trunk muscles [17]. In the *Myf5^{nGFP/+}* mouse, the nGFP (nuclear GFP) reporter is expressed in a subpopulation of satellite cells [18], and the two *Myf5^{Cre}* alleles allow the detection of about 90 %, but not all satellite cells in young adult mice [19–21, 10, 22]. Another GFP reporter line, *Nestin-GFP*, has been used to isolate satellite cells [23]. New genetic tools will

continue to emerge allowing isolation of subpopulations of muscle stem cells in quiescence and their purification on further step down the myogenic differentiation program.

In addition to isolation of muscle stem cells by flow cytometry, preparation of isolated single myofibers remains an efficient method to isolate and follow individual satellite cells and their fate [24, 25]. This method provides the advantage of isolating satellite cells within their niche, with minimal artificial stress induced by other methods such as FACS. However, the amount of cells that are collected at quiescence is limited, and isolation of activated or proliferating satellite cells needs to be performed *ex vivo*.

In this chapter, we describe the isolation of satellite cells by FACS where these cells are marked by a fluorescent reporter such as the previously described *Tg:Pax7-nGFP* reporter mice [11]. The benefit of fluorescent reporter mice is the high yield of satellite cells collected in a reasonable amount of time, without the use of antibody staining for surface markers that can be compromised following enzymatic treatment. Muscle stem cells isolated by enzymatic treatments remain functionally competent and are able to proliferate and generate robust myogenic fibers *in vitro* and *in vivo*. Transplantation of a single or a population of satellite cells is able to contribute to regenerating myofibers and self-renew efficiently [18, 26, 27].

However, in many cases, the crossing of a fluorescent reporter with other genetically modified mice is time-consuming and cumbersome; therefore, the use of surface markers becomes mandatory. Currently, there is no single cell surface marker that can be universally used to identify quiescent or activated satellite cells. Most of the strategies rely on the combination of at least two positive markers for satellite cells and several negative markers for exclusion of non-myogenic cells. For example, the protocol proposed in this chapter is based on two cell surface markers: $\alpha 7$ -integrin and CD34 [21, 28]. Other examples reported previously use different cell surface marker combinations including CXCR4, VCAM, SM/C2.6 [29–32], or Syndecan4 [33].

It should be noted that a major drawback of stem cell isolation and FACS is the stress that is imposed on cells, the consequences of which remain unknown. These have yet to be quantified but should be taken into account particularly in assays such as the measure of metabolic activity. Furthermore, the quiescent status of stem cells is immediately compromised as soon as muscles are isolated. Therefore, satellite cells isolated on single fibers or by FACS have already initiated the G0/G1 transition [34]. To overcome the problem of stress induced by FACS to the cells, an alternative method relies on magnetic activated cell sorting (MACS) [35].

For human muscles, a different strategy is required as some of the cell surface receptors of quiescent satellite cells are different. For example, the cell surface receptor CD34 is not expressed in

quiescent human satellite cells [36]. Instead, CD56 is expressed in some quiescent satellite cells and more robustly in activated cells derived from cultured satellite cells. Therefore, CD56 is commonly used to enrich the human myogenic cells following expansion in culture. Different approaches have been developed recently to isolate human muscle stem cells, from manually dissected muscle fiber fragments [37] to FACS using $\alpha 7$ -integrin. CXCR4 or CD29 cell surface receptors are also used as positive markers [38–40].

Here, we describe two approaches for the isolation of satellite cells:

1. *Isolation of mouse skeletal muscle stem cells with collagenase D and trypsin:* Collagenase D cleaves native collagen. It has a high collagenase activity and a low contaminating tryptic activity. Trypsin is a serine endopeptidase; it cleaves peptide bonds at the carboxylic side of the basic amino acids Arg and Lys. Due to the rapid and broad range of action of the trypsin, this procedure should not be employed extensively if the isolation of muscle stem cell requires the recognition of cell surface antigens. However, this protocol has proven to be efficient for skeletal muscles when fluorescent reporter mice are used to mark satellite cells.
2. *Isolation of mouse muscle satellite cells with collagenase A and dispase II:* Collagenase A degrades native collagen and has a balanced ratio of contaminating enzyme activities. Dispase II is a neutral protease that hydrolyzes the N-terminal peptide bonds of nonpolar amino acid residues. This enzyme combination allows an efficient release of satellite cells from the tissue and it minimizes the cleavage of cell surface receptors necessary for immunodetection and cell sorting [41].

2 Materials

2.1 Isolation of Mouse Muscle Stem Cells with Collagenase D and Trypsin (C/T)

1. Dulbecco's modified Eagle's medium (DMEM, Life Technologies, ref. 31966021) with penicillin/streptomycin (Life Technologies, ref. 15140122).
2. Trypsin stock solution (Life Technologies, ref. 15090-046). Make 15 ml tube aliquots of 2.5 % trypsin stock solution and store at -20°C .
3. Collagenase D stock solution (Roche, ref. 1108882001). Collagenase D powder is resuspended with cold DMEM to make a 1 % stock solution (250 ml DMEM for 2.5 g collagenase D). Make 15 ml tube aliquots and store at -20°C .
4. DNase I stock solution (Roche, ref. 11284932001). DNase I is resuspended in cold DMEM to make a 10 mg/ml stock solution. Make aliquots and store at -20°C . Use at a final concentration of 0.1 mg/ml (*see Note 1*).

5. Fetal bovine serum (FBS): serum is used to block trypsin activity once digestion is complete. We do not heat inactivate FBS.
6. Collagenase D/Trypsin working solution (C/T): add 4 ml of collagenase D stock solution and 2 ml of trypsin stock solution to 44 ml of DMEM to obtain final concentrations of 0.08 % collagenase D and 0.1 % trypsin. Add 50 μ l of stock DNase I solution to the final 50 ml of C/T solution. This solution is prepared extemporaneously (*see* **Note 2**).
7. Cell strainers; 100, 70 (Miltenyi Biotec, ref. 130-098-463, 130-098-462), and 40 μ m (BD Falcon, ref. 352340).
8. Dissecting tools are cleaned and sterilized by autoclaving or 70 % alcohol.

2.2 Isolation of Mouse Muscle Stem Cells with Collagenase A and Dispase II (C/D)

1. Dispase II (Roche, ref. 04942078001): the number of U/mg is provided on each commercial bottle. Dispase II is weighed extemporaneously and used at a final concentration of 2.4 U/ml (*see* Subheading 2.2, **item 4**).
2. Collagenase A (Roche, ref. 11088793001): collagenase A is weighted extemporaneously to be used at a final concentration of 0.2 % (*see* Subheading 2.2, **item 4**).
3. Hank's Balanced Salt Solution (HBSS) (Life Technologies, ref. 24020091) with penicillin/streptomycin (Life Technologies, ref. 15140122).
4. For 10 ml of working collagenase A/dispase II solution (C/D): weigh 20 mg of collagenase A and the appropriate quantity of dispase II (e.g., 24 mg for a dispase II at 1 U/mg), and resuspend in 10 ml of HBSS. DNase I (stock 10 mg/ml; *see* Subheading 2.1) is added to the solution to a final concentration of 0.1 mg/ml. The solution is filtered through a 22 μ m filter and kept at room temperature.
5. Washing solution: HBSS with penicillin/streptomycin and 2 % fetal bovine serum.

3 Methods

3.1 Isolation of Mouse Muscle Stem Cells with Collagenase D and Trypsin (C/T)

1. Before dissection, fill 50 ml Falcon tubes with 5 ml of FBS, and place in an ice bucket. Place 100 and 70 μ m cell strainers on top.
2. Skeletal muscles are dissected with small scissors from body parts of interest (*see* **Note 3**). Exclude as much as possible adipose tissue (white fat), nerves (like the sciatic nerve running through the hind limb), and tendons. Collect the dissected muscles in a small volume (1 ml) of DMEM in a petri dish (*see* **Note 4**).

3. Before mincing the tissue, remove the excess DMEM with a pipette or by placing the petri dish on an angle. Removing the excess of liquid will facilitate mincing the tissue.
4. Use fine dissecting scissors (Moria, ref. 4878) to mince the tissue until a slurry forms with no more large muscle pieces (*see Note 5*).
5. Transfer minced tissue to a 50 ml tube filled with DMEM-Pen/Strep. Mix by inverting the 50 ml tube several times to resuspend the tissue. Leave the tube on ice for 10 min to allow the muscle to sediment, while the fat tissue will stay in suspension. When all the muscle tissue is sedimented, remove the excess of DMEM (*see Note 6*).
6. Resuspend the sedimented tissue with 10 ml of collagenase D/trypsin working solution (*see Note 7*).
7. Incubate the tube at 37 °C for 25 min in an agitating water bath. To increase the surface between the tissue and enzymes, tubes are incubated in a horizontal position with a gentle agitation (120 rpm). This is a critical step that can impact on the yield of satellite cells.
8. After 25 min of incubation, stop the agitation, and place the tubes in a tube holder in a vertical position to allow tissue sedimentation for 3–5 min.
9. Collect the supernatant by decanting or with a pipette.
10. Filter the supernatant through cell strainers 100 and 70 μm consecutively. The filtered supernatant is collected in 50 ml tubes prepared earlier with serum on ice (*see Note 8*).
11. Resuspend the tissue with 10 ml of collagenase D/trypsin working solution. Repeat **steps 6–10** until all tissue is digested (*see Note 9*).
12. Centrifuge all collected supernatant tubes for 10 min at $50 \times g$ at 4 °C. Transfer the supernatant in a new 50 ml tube, and discard the pellet (*see Note 10*).
13. Centrifuge the collected supernatant for 15 min at $550 \times g$ at 4 °C.
14. Discard the supernatant by decanting or with a pipette. Keep the pellet.
15. Wash the pellet by gently resuspending with 40 ml of cold DMEM.
16. Centrifuge for 15 min at $550 \times g$ at 4 °C (*see Note 11*).
17. Discard the supernatant by decanting or with a pipette. Keep the pellet.
18. Resuspend the pellet in 40 ml of cold DMEM, and filter it through a 40 μm cell strainer.
19. Centrifuge for 15 min at $550 \times g$ at 4 °C.

20. Resuspend the pellet in cold DMEM with 2 % FBS. The cell suspension is ready to use for sorting. Keep cells on ice until used.
21. Cells can be collected out of the FACS in DMEM/2 % FBS for further molecular analysis and cell culture or directly in lysis buffer for RNA preparation. If a precise number of cells are required for the following experiments, we recommend to verify the actual number of cells collected by FACS with a hemocytometer (e.g., Malassez counting chamber).

3.2 Isolation of Mouse Muscle Stem Cells with Collagenase A and Dispase II (C/D)

1. Repeat **steps 1–5**, from Subheading 3.1, replacing DMEM by HBSS.
2. Resuspend the sedimented tissue with 10 ml of collagenase A/dispase II working solution (*see Note 12*).
3. Incubate the tube at 37 °C for 90–120 min in an agitating water bath. To increase the surface between the tissue and enzymes, tubes are incubated in a horizontal position with a gentle agitation (*see Note 13*). This is a critical step that can impact on the yield of satellite cells.
4. When muscles are fully dissociated, stop the digestion.
5. Add 30 ml of HBSS to the cell suspension, and filter consecutively through 100, 70, and 40 µm filters.
6. Centrifuge for 15 min at $550 \times g$ at 4 °C.
7. Remove the supernatant. At this point, the pellet can be processed for antibody staining (*see Subheading 3.3*) or resuspended in washing solution if the sample is directly used for FACS (*see Note 14*).

3.3 Cell Surface Receptor Staining for Isolation of Satellite Cells by Flow Cytometry

We recommend performing cell surface receptor staining following the collagenase A/dispase II protocol (*see Subheading 3.2*) which preserves to a greater extent the surface antigens:

1. A set of controls is required to establish a correct gating of cell populations during FACS acquisition.
Recommended controls for this protocol are the following; 1/30 of the cell preparation is necessary to carry out each of the controls (*see Note 15*):
 - (a) Negative control: keep a small aliquot of the sample unstained. It is used to set up the voltage of the lasers and the threshold between positivity and negativity of different fluorochrome-labeled cell populations.
 - (b) Single conjugated antibody staining: perform an individual staining for each fluorescent dye-coupled antibody on a small fraction of the sample. These staining are necessary to perform the compensation. Compensation is a technique used to eliminate false signal that results from spectral

overlap between fluorescent dyes when used in multicolor staining panels.

- (c) Fluorescence minus one (FMO) staining: FMO control is used to properly interpret flow cytometry data. In this control, all conjugated antibodies are included except one which is necessary to discriminate your final population (in the proposed protocol, we excluded CD34).
 - (d) Isotype control: fluorochrome-coupled antibodies with the same isotype as the primary antibodies used for the surface staining are important to confirm the specificity of primary antibody binding and help to assess the level of background staining (*see Note 16*).
2. Prepare the mix of conjugated antibodies at the indicated concentrations (*see Table 1*) in 500 μ l of washing solution (HBSS/2 % FBS).
 3. Resuspend the cell pellet in the conjugated antibody mix, and incubate on ice for 30 min (*see Note 17*).
 4. Add three volumes of washing solution and centrifuge for 15 min at $550 \times g$ at 4 °C.
 5. Remove the supernatant and resuspend the cells in 2 ml of washing solution.
 6. Centrifuge for 15 min at $550 \times g$ at 4 °C.
 7. Resuspend the pellet in an adequate volume of washing solution (100–500 μ l), and keep the cells on ice until sorting.

3.4 Profiles of Satellite Cells Isolated by FACS

FACS profiles of quiescent and activated satellite cells isolated from *TgPax7-nGFP* mice are shown in Fig. 1. Quiescent satellite cells are clustered as a homogeneous population, characterized by a small size (FSC) and a low granularity (SSC). However, activated satellite cells analyzed 40 h after cardiotoxin injury of the muscle present a larger and heterogeneous size with an increased granularity.

Table 1
Dilution of primary antibodies for cell surface staining of quiescent mouse muscle stem cells

| Antibody | Clone | Conjugated dye | Dilution | Isotype | Source |
|----------------------|----------------------|----------------|----------|---------------------|---------------|
| CD45 | 30-F11 | PE-Cy7 | 1/400 | Rat IgG2b, κ | eBioscience |
| CD31 | MEC 13.3 | PE | 1/50 | Rat IgG2a, κ | BD Pharmingen |
| Sca-1 | D7 | PE-Cy7 | 1/100 | Rat IgG2a, κ | eBioscience |
| CD34 | RAM34 | eFluor 450 | 1/20 | Rat IgG2a, κ | eBioscience |
| $\alpha 7$ -Integrin | $\alpha 7$ -Integrin | Alexa 647 | 1/1000 | Rat IgG2b | AbLab |

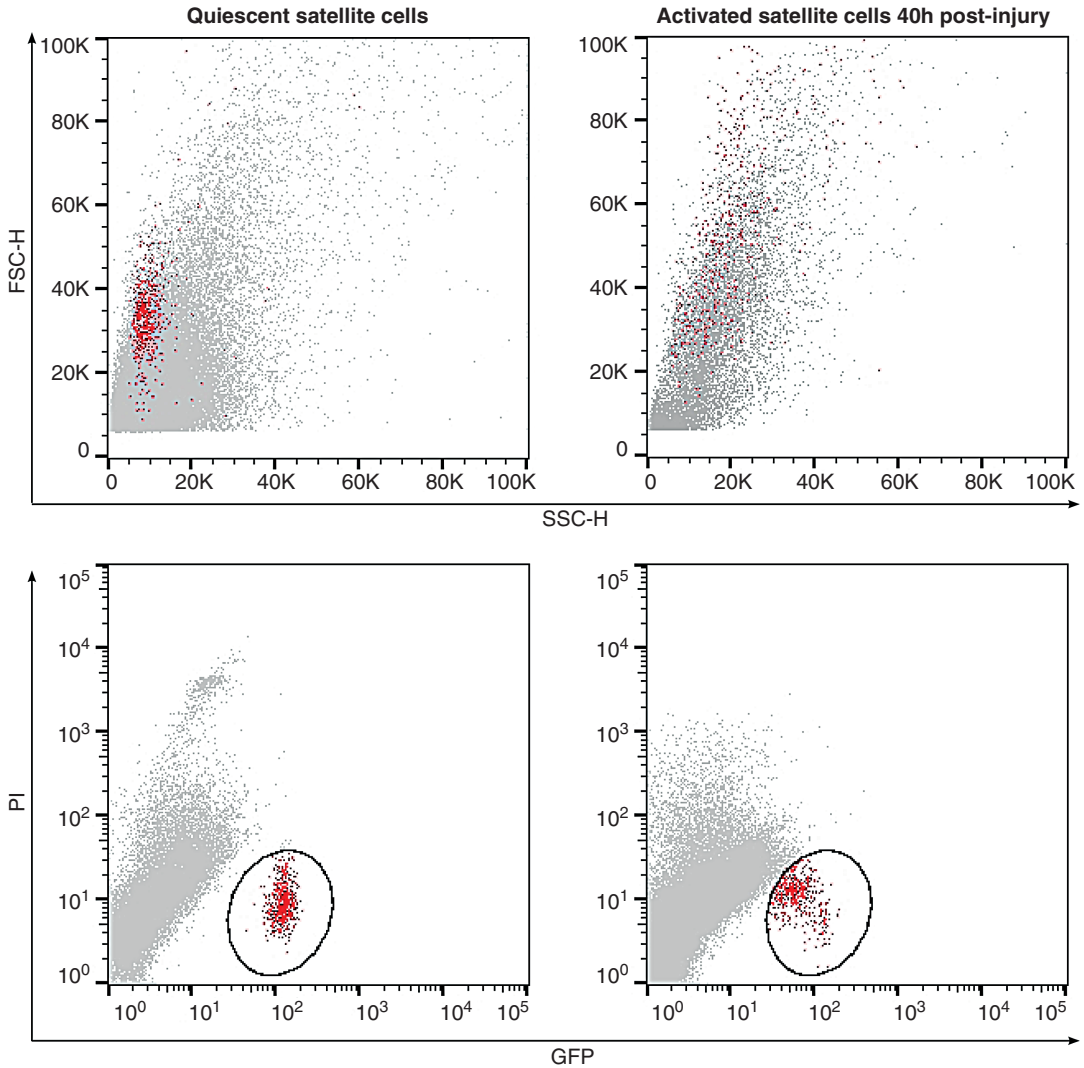


Fig. 1 FACS profiles for isolation of quiescent and activated muscle stem cells from *TgPax7-nGFP* muscles. The GFP⁺ cell population (red) was backgated on scatter gates to highlight small size and low granularity of quiescent muscle stem cells, while activated muscle stem cells are heterogeneous in size and in granularity. Fluorescence-activated cell sorting was performed on an Aria III (BD Biosciences) or MoFlow (BD Biosciences), and data were analyzed using FlowJo (BD Biosciences)

Cells are displayed on a plot with GFP on the X axis (FITC-A, fluorescein isothiocyanate area) and propidium iodide on the Y axis (PE-Texas Red, phycoerythrin area) to detect dead cells. From our experience, the proportion of dead cells within the Pax7-nGFP⁺ cell population after the digestion protocol is very low, generally not exceeding 0.05 %. Representative yields of Pax7-nGFP⁺ cells collected from different muscles are presented in Table 2.

As described in this chapter, muscle stem cells can be isolated by flow cytometry using cell surface markers. Figure 2 shows the

Table 2
Representative number of Pax7-nGFP⁺ cells extracted with collagenase D/trypsin from *TgPax7-nGFP* mouse muscles from different anatomical locations. These values are indicative of the relative cell numbers; actual numbers can be higher

| | Pax7-nGFP ⁺ sorted cells/muscle |
|-----------------|--|
| EDL | 2000–3000 |
| TA | 4000–7000 |
| Soleus | 8000–9000 |
| Quadriceps | 30,000–40,000 |
| Leg (hind limb) | 120,000–180,000 |
| Abdomen | 100,000–120,000 |
| Diaphragm | 20,000–30,000 |
| EOM | 6000–8000 |
| Tongue | 30,000–50,000 |
| Masseter | 20,000–30,000 |
| Back | 22,000–30,000 |

gating strategy, starting by FSC and SSC, followed by selection of the negative population for CD45, Sca-1, and CD31 used to exclude non-myogenic cells, and finally the selection of the double-positive population $\alpha 7$ -integrin⁺ and CD34⁺ chosen as positive myogenic markers. As indicated, a sample treated with all conjugated antibodies except one (fluorescence minus one (FMO)) is necessary to set and select the appropriate population of interest.

Both sets of enzymes presented in this protocol, collagenase D/trypsin or collagenase A/dispase II, can be efficiently used to collect muscle stem cells from transgenic models such as *Tg:Pax7-nGFP* (Fig. 3a, Table 3). However, the cell surface receptor staining strategy, due to additional steps for the staining, the required controls, and the gating strategy, reduces greatly the amount of cells collected compared to the genetically marked muscle stem cells (Table 3). Table 3 shows the susceptibility of cell surface receptors to enzymatic treatment by the collagenase D/trypsin digestion (mainly due to the trypsin activity) compared to the cells extracted with collagenase A/dispase II. The endothelial cell receptor, recognized by CD31, is particularly susceptible to proteolytic cleavage.

Fig. 2 (continued) stained with $\alpha 7$ -integrin, which recognizes all satellite cells but also some non-myogenic cells. To enrich for satellite cells, the suspension is double stained with CD34. These double positive $\alpha 7$ -integrin⁺/CD34⁺ cells correspond largely to satellite cells

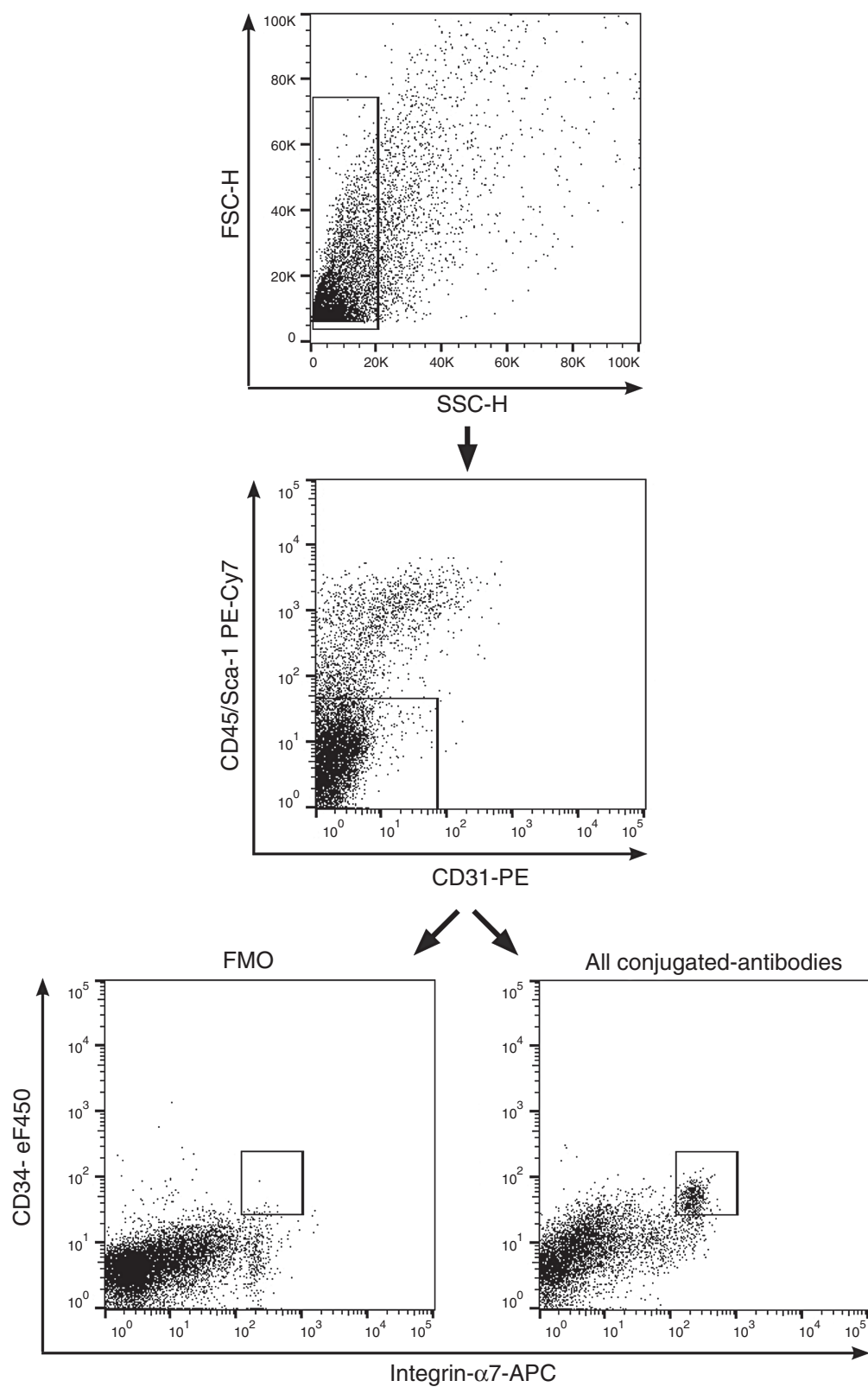


Fig. 2 FACS profile of $\alpha 7$ -integrin⁺CD34⁺ satellite cells. The strategy described here for isolating satellite cells with cell surface markers relies on the elimination of non-myogenic cells: CD45⁺ hematopoietic cells, CD31⁺ endothelial cells, and Sca-1⁺ non-myogenic cells including endothelial cells and fibroblasts. Satellite cells are

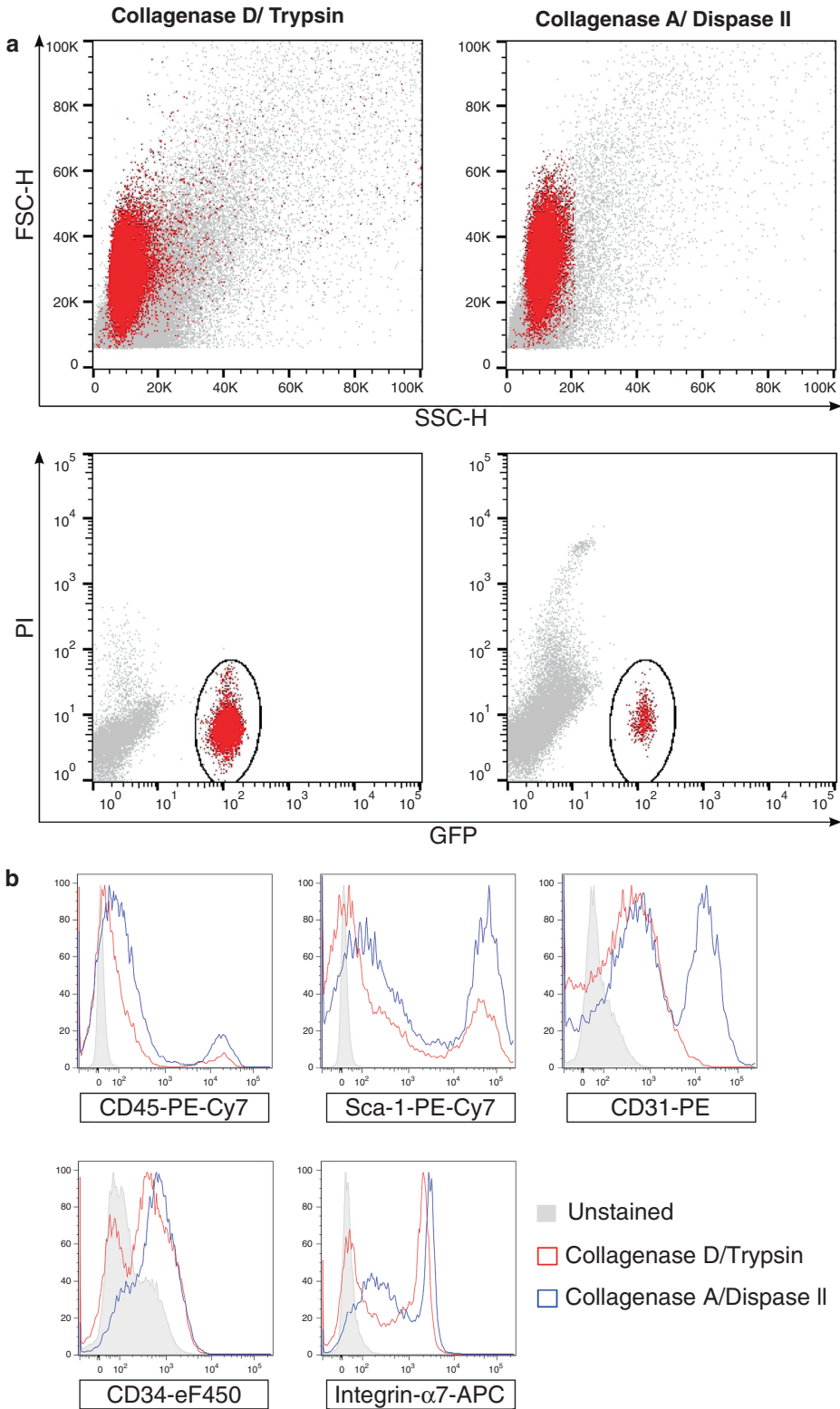


Fig. 3 FACS profiles following enzymatic digestions of skeletal muscles. **(a)** Profile of isolated muscle stem cells from collagenase D/trypsin or collagenase A/dispase II enzymatic treatments does not differ when isolating

Table 3

Comparison of the number of cells collected following collagenase D/trypsin or collagenase A/dispase II digestion. For a given muscle (here, quadriceps), the number of Pax7-nGFP⁺ cells collected with the two sets of enzymes is similar. However, supplementary steps required for antibody staining of cell surface receptors and FACS diminish greatly the number of cells collected

| | Number of Pax7-nGFP ⁺ cells/quadriceps | Number of Pax7-nGFP ⁺ cells/1 leg (hind limb) | Number of CD34 ⁺ /Itg7 ⁺ cells/1 leg (hind limb) |
|---|--|---|--|
| Collagenase D/trypsin FACS on GFP expression | 32,000 | 170,000 | n.d. |
| Collagenase A/dispase II FACS on GFP expression | 35,000 | n.d. | n.d. |
| Collagenase A/dispase II + FACS on cell surface markers | n.d. | n.d. | 60,000 |

4 Notes

1. DNase I is required especially when preparing muscle stem cells from injured muscle, where a high level of inflammatory cells and dead cells release their DNA during the extraction protocol. This released DNA will interfere later with cell sorting.
2. Prepare working collagenase D/trypsin solution with room temperature DMEM. This minimizes the time to reach 37 °C during digestion. Keep stock solutions on ice, especially trypsin which can self-degrade at 37 °C.
3. The choice of which skeletal muscles are to be used for muscle stem cell isolation has to be considered given the reported heterogeneity among skeletal muscle groups. Head and trunk muscles have different development origins and are governed by distinct genetic networks. Limb and trunk muscles originate from different somitic regions. Moreover, skeletal muscles support different physiological functions (slow or fast twitch) and different metabolisms (oxidative or glycolytic) [42, 43].

Fig. 3 (continued) Pax7-nGFP⁺ cells. **(b)** Histogram of single antibody staining of muscle stem cells isolated with the two different sets of enzymes shows the susceptibility of some cell surface receptors to collagenase D/trypsin treatment compared to collagenase A/dispase II treatment. The CD31 receptor, and to a lesser extent Sca-1, displays a high susceptibility to trypsin digestion

4. If several mice need to be dissected, perform dissections in DMEM, and keep on ice before proceeding to the following steps.
5. Fine curved scissors (Moria, ref. 8142A) are more appropriate than straight fine scissors.
6. This step can be omitted if the starting material is inferior to 1 g of tissue.
7. 10 ml of collagenase D/trypsin working solution is appropriate for 2 g of dissected muscles. Increase proportionally the amount of collagenase D/trypsin working solution with the amount of dissected muscles (20 ml of C/T for 4 g of muscle).
8. Collection of supernatant can be performed directly through 40 μ m cell strainer if starting material is inferior to about 0.3 g, for example, for one or two *tibialis anterior* muscles.
9. Four rounds of digestion are usually enough to digest 2–4 limb muscles. If large pieces of tissue are still visible after the third round of digestion, the pellet can be transferred into a petri dish and minced with scissors before being resuspended in fresh collagenase D/trypsin working solution.
10. This first gentle centrifugation pellet contains essentially debris, whereas satellite cells remain in the supernatant. This step should not be performed if the starting material is inferior to about 0.5 g of tissue.
11. If the pellet is still large, a second wash with DMEM can be performed as mentioned in **steps 14** and **15**.
12. A maximum of 2.5 g of tissue should be digested per tube otherwise the suspension becomes too viscous.
13. If digesting a large amount of material, the digestion can be split in two rounds of 45 min, with addition of fresh enzyme solution to the pellet as described in Subheading 3.1.
14. If antibody staining is not considered, repeat washing by adding 30 ml of HBSS, followed by centrifugation of 15 min.
15. If a small sample, for example a TA, needs to be processed for antibody staining to isolate muscle stem cells, controls can be performed on a muscle from other locations (any other limb muscle).
16. For convenience, controls performed in small volumes of sample can be easily performed in V-bottom 96 well plates (Corning, ref. 3897).
17. Alternative cell surface markers for quiescent satellite cell staining and cell surface markers for non-myogenic cells (Table 4).

Table 4**Alternative cell surface markers for quiescent satellite cell staining and cell surface markers for non-myogenic cells**

| Positive cell surface satellite cell markers | |
|--|----------------|
| SM/C2.6 ^a | [31] |
| VCAM ^a | [29, 30] |
| CXCR4 | [32] |
| Exclusion markers | |
| CD11b | Macrophage |
| Ter119 | Red blood cell |

^aSM/C2.6 antigen corresponds to VCAM

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