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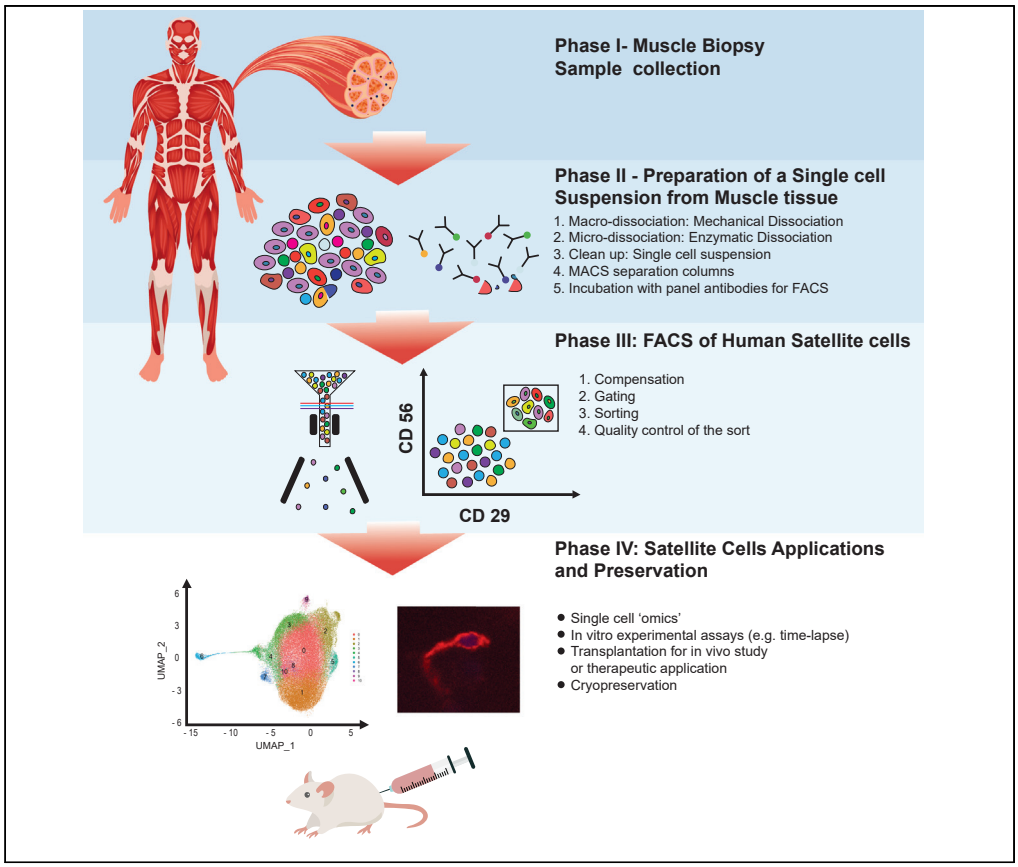
Purification and preservation of satellite cells from human skeletal muscle



Regeneration and repair of skeletal muscle is driven by tissue-specific progenitor cells called satellite cells, which occupy a minority of the cells in the muscle. This protocol provides researchers with techniques to efficiently isolate and purify functional satellite cells from human muscle tissue. The proven techniques described here enable the preparation of purified and minimally altered satellite cells for *in vitro* and *in vivo* experimentation and for potential clinical applications.

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HIGHLIGHTS

Techniques are described for efficient isolation of human satellite cells

Quiescent and activated satellite cells are purified using surface markers

Purification involves minimal alteration compared to culture or activation

Purified satellite cells faithfully represent the natural state for applications

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Purification and preservation of satellite cells from human skeletal muscle

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SUMMARY

Regeneration and repair of skeletal muscle is driven by tissue-specific progenitor cells called satellite cells, which occupy a minority of the cells in the muscle. This protocol provides researchers with techniques to efficiently isolate and purify functional satellite cells from human muscle tissue. The proven techniques described here enable the preparation of purified and minimally altered satellite cells for *in vitro* and *in vivo* experimentation and for potential clinical applications.

For complete details on the use and execution of this protocol, please refer to Barruet et al. (2020) and Garcia et al. (2018).

BEFORE YOU BEGIN

Ethical biomedical research

Timing: 1–3 months

1. Ensure that all protocols and procedures that require human subjects are conducted under the approval of the Committee on Human Research at your institution and corresponding local and national regulating authorities.
2. If doing animal research, make sure all the protocols are updated and have approval from the Animal Research Protection agencies at your institution. All procedures involving experimental animals should be performed in accordance with relevant institutional and national regulations and within dedicated experimental animal care facilities.

Prepare equipment

Timing: 1–2 weeks ahead

3. A flow cytometer sorter with capacity of at least 4 different wavelength lasers is required. We use the BD FACS Aria II at the UCSF Flow core facility.
4. Book the flow cytometer sorter for an appropriate length of time. Bear in mind that it usually takes 30 min to 60 min for compensation and gating. Calculate duration of the sort according to the size of the sample, with at least 30 min for every gram of muscle.

⚠ CRITICAL: Details pertaining to the setup and proper use of the sorter are out of the scope of this protocol but vital for successful sorting.

Prepare cell culture media

Timing: 1–2 weeks ahead

Note: It is of utter importance to ensure sterility throughout the process, starting from the preparation of the cell culture media, as follows. Prepare 500 mL of each of the following basic media, according to the Table 1. The media can be stored at 4°C for short-term use (1–3 months).

Table 1. Preparation of media and buffers

For a 500 mL final volume	Base	FBS	Pen/Strep 100×	EDTA 0.5 M	1 M HEPES
Muscle collection media	345 mL DMEM high glucose	150 mL	5 mL	–	–
Digestion media	445 mL DMEM high glucose	50 mL	5 mL	–	–
PBS with 2% FBS	490 mL 1× PBS	10 mL	–	–	–
MACS buffer	483 mL × PBS	10 mL	–	2 mL	5 mL

Usually you will need 30 to 60 mL of Muscle collection media for one muscle sample up to 10 g. Afterwards, you will need 10 mL of digestion media with collagenase for each 1 g of muscle. Have a minimum of 200 mL of MACS and PBS with 2% FBS per sample ready to use.

5. Muscle collection Media: Dulbecco's Modified Eagle Medium (DMEM) with high glucose, 30% FBS, 1% Penicillin/Streptomycin.
6. Digestion media: Dulbecco's Modified Eagle Medium (DMEM) with high glucose, 10% FBS, 1% Penicillin/Streptomycin.
7. MACS buffer: PBS, 10% FBS + 2% EDTA (0.5 M, Ph: 8.0 purchased, already sterile and filtered) + 10 mM HEPES (1 M, pH 7.4) and centrifuged 5 min at 4°C at 2,000 rpm (500 RCF) to diminish bubbles. Keep it ice cold or refrigerated until use.

Human donor samples

Timing: the day(s) before

8. As biopsies are obtained from individuals undergoing surgery, close coordination with the surgical team is required for possible donor opportunities.

⚠ CRITICAL: Written informed consent should be obtained from all subjects and properly filed according to the institution's regulations.

Note: The type of biopsy is given by the named muscle of origin and can be from the craniofacial or from the body skeletal muscle. The size of the biopsy varies given each individual situation; biopsies typically range between 1 g and 20 g, and these sizes can be processed by one person. Large samples are usually derived from waste muscle harvested during flap operations and are less frequently obtained than smaller samples. More rarely, whole cadavers might be donated to research, including deceased individuals with rare muscle diseases, requiring a highly coordinated team to receive the body rapidly enough after death and a minimum of two or three people in the lab to process the muscles of the body in an efficient amount of time.

Timing: the day of the isolation of Hu-MuSC

⚠ CRITICAL: Use freshly prepared digestion media with collagenase to optimize tissue enzymatic dissociation.

9. Add collagenase to the digestion media:
   a. Dilute a vial of collagenase (50 mg- lyophilized powder), in a 50 mL conical tube filled with digestion media for a concentration of 1 mg/mL, vortex well.
   b. Filter using a 0.22 μm filter/vacuum. 10 mL of digestion media with collagenase are needed to digest 1 g of muscle. Prepare the amount of digestion media according to the initial weight of your sample.

Note: Collagenase type XI is a critical reagent to break up the collagen and connective tissue and release the Hu-MuSC from their niche within the basal lamina and the myofibers. We have not tested other collagenases for this purpose.

10. Keep digestion media and trypsin 0.25% in the heating rocks.
11. Prepare fresh DNase media the day of the digestion as follows: DNase 500 μL aliquots are kept frozen at −20°C and are only thawed before use. 500 μL of DNase (stock 10 mg/mL) in 9.5 mL DMEM for a final concentration of 10 μg/mL.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CD45 human metal beads	Miltenyi Biotec	130-045-801
CD31 human metal beads	Miltenyi Biotec	130-091-935
CD31 Human 450	Ebioscience	48-0319-42
CD34 human 450	Ebioscience	48-0349-42
CD45 human 450	Ebioscience	48-0459-42
CD56 human APC-vio	Miltenyi Biotec	770 130-100-694
CD29 human APC	Ebioscience	17-0299-42
CD29 human FITC	Ebioscience	11-0299-42
CXCR4 human APC	Ebioscience	17-9999-42
CXCR4 human PE	Ebioscience	12-9991-82
Sytox blue	Invitrogen	S34857
LD column for MACS	Miltenyi Biotec	130-042-901
MACS multistand	Miltenyi Biotec	130-042-303
PAX7 antibody for IF (from mouse)	Developmental Studies Hybridoma Bank (DHSB)	n/a
Purified mouse anti-MyoD	BD Pharmigen	554130
Chemicals, peptides, and recombinant proteins		
Collagenase XI	Sigma-Aldrich	C9697
DNase	Invitrogen	18047-019
DMEM with high glucose	Gibco	11965-092
Insulin-transferrin-selenium (ITS)	Gibco	41400-045
Fetal bovine serum	Gibco	16141-061
ACK lysis buffer	Quality Biological	118-156-101
Penicillin/streptomycin	Quality Biological	120-095-721
DMSO	R&D Systems	3176
EDTA 0.5 M, pH 8.0 sterile solution	Bioworld	40520000-1
HEPES 1 M, pH 7.4 sterile, DNase RNase free	Bioworld	40820008-1

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Vectashield	Vector laboratories	H-1200-10
PBS sterile, filtered no calcium no magnesium	Gibco	14190-144
0.25% trypsin with EDTA	Life Technologies	25200072
Software and algorithms		
FACS DIVA	BD Biosciences	n/a
FLOW JO	LLC	n/a
Other		
13 gauge needle	Cadence Science	4093
19 gauge needle	BD Precision Glide Needle	305187
50 mL or 60 mL syringes	BD	309653
20 mL syringes	BD	302830
Stainless steel surgical blade	Bard-Parker	371610
0.22 µm filter	Corning	431215
Sterile 50 mL disposable vacuum filtration system 0.22 µm	Millipore	SE1M179M6
50 mL tubes	Olympus plastics	28-108
5 mL polystyrene tube with cell strainer cap	Falcon	352235
Cryostorage tubes	Corning	431386
Microcentrifuge tubes 1.5 mL low retention	Fisher Scientific	02-681-320
ART 200 µL pipette tip wide-bore genomic sterile	Molecular Bioproducts	2069G
Cell filter strainer 40 µm	Corning	431750
Microcentrifuge tubes 1.5 mL low retention	Fisherbrand	02-681-320

STEP-BY-STEP METHOD DETAILS

Phase I. Muscle biopsy: collection of the sample

Timing: the day before or the day of isolation, duration: 30 min

⚠ CRITICAL: It is very important to ensure that the muscle biopsy is handled under sterile conditions and is immersed in muscle collection media as soon as possible after removal from the body and kept on ice or at 4°C at all times to prevent tissue contamination and degradation.

1. Collect the sample and place in cold Collection media.
2. Keep the muscle biopsy in 50 mL conical tube(s) filled with 25 mL cold muscle collection media and keep on ice at all times.
3. Weigh the muscle sample. Tare an empty 50 mL conical tube and using sterile forceps, carefully place the muscle sample inside to record the weight. Annotate for future calculations.

   Note: The sample should be weighed as soon as possible. If you wait overnight to weigh the sample, the weight might not be accurate due to accumulation of media in the muscle. Record information such as the type of muscle, age of the individual, and gender. Additional information like history of diseases and medications taken could be useful, depending on the purpose and approval by the study Committee on Human Research.

   Note: A secure sample database with the desired sample information is useful. Comply with human subject de-identification as described in your approved protocol.

4. This step is facilitated by using a 4× or 10× magnification lens. Remove connective and adipose tissue and any cauterized or devitalized tissue contaminants from the muscle sample. Remove any other contaminant (such as surgical foreign bodies -as in the Figure 1) or necrotic parts (black or

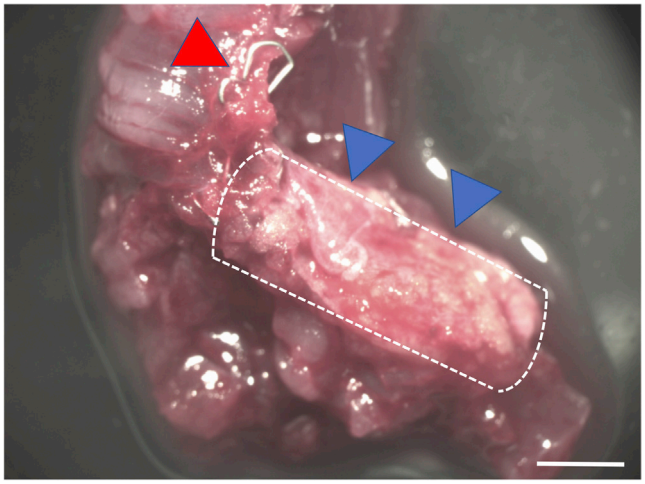

Figure 1. Muscle biopsy from a gracilis muscle

First clean-up of the muscle biopsy from a gracilis muscle of a 54-year-old female by precise dissection of visible connective and fat tissue, depicted here with the blue arrow, taking care to minimally disrupt the adjacent muscle fibers. The red arrow indicates a surgical metallic foreign body that has to be removed as well as any other gross contaminant. Scale bar: 0.5 cm.

burned) from the muscle. The connective tissue looks white and sometimes transparent and care should be taken to dissect it from the muscle without severing the muscle fibers as much as possible.

Optional: At this point you may reserve a fraction of the muscle sample for other experiments such as for histology, for instance.

5. The sample destined to FACS should be placed in a conical tube(s) with 40 mL of ice cold muscle collection media and kept at 4°C.

Pause point: At this point you may proceed with the isolation protocol or continue the next day. Previous work in our laboratory has shown that is possible to isolate Hu-MuSC for at least a few days after collection; however proceeding to the isolation process within 18 h after sample collection will minimize Hu-MuSC activation and tissue decay and will maximize yield.

Note: Keep the sample on ice or refrigerated at 4°C **at all times** and work efficiently to aid cell viability after sorting. Avoid cutting or manipulating the muscle sample too much in order to minimize injury to the fibers and subsequent activation of the Hu-MuSC.

Phase II. Preparation of a single-cell suspension from muscle tissue

Timing: total duration of phase II using MACS columns, 6 h+; total duration of this phase without the MACS, 4–5 h

Phase IIa. Macro-dissociation: mechanical dissociation of the muscle

Timing: 10 min/g of tissue

Note: Ensure a biological safety laminar flow cabinet/cell culture cabinet class II or III to maintain sterility throughout the entire process.

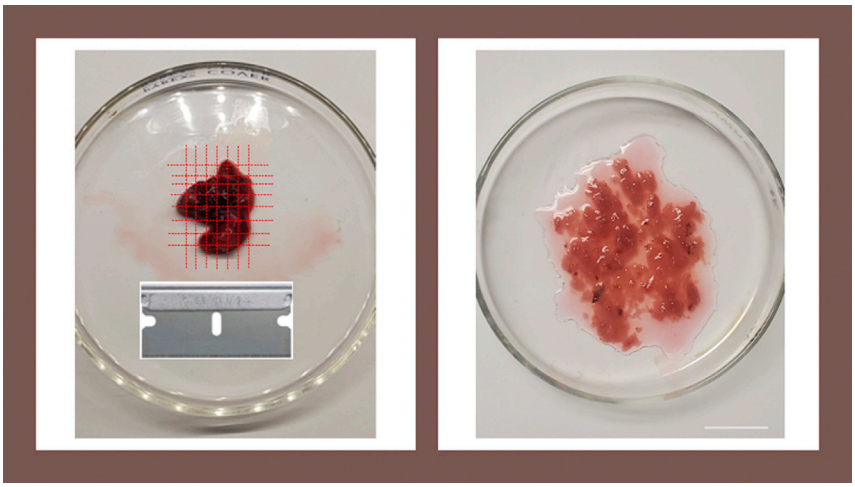

Figure 2. Macro-dissociation

Mechanical dissociation of the muscle sample is done using a box cutting blade in a 10 mm Petri dish to sharply and cleanly cut pieces in <1 mm^2 without smashing or crushing the tissue. Scale bar: 2 cm.

6. The goal is to go from whole muscle to minced muscle to clumps of cells by meticulous mechanical dissociation using blades, syringes, and washes. It is important to keep the muscle immersed in prewarmed digestion media, never letting the tissue dry. Minimize shear cell stress by avoiding mashing or grinding the tissue or pipetting too fast.
7. Prewarm the digestion media with collagenase (10 mL for each gram of muscle) and trypsin 0.25% (3 mL for every gram of tissue) in heating rocks or water bath at 37°C.

   Note: If processing more than one biological sample (different donors) at the same time, label clearly throughout the process and use separate dissection equipment for each sample, including blades, syringes, tubes, pipettes, Pasteur pipettes, and any other equipment that comes into contact with the tissue to avoid contaminating the samples. Dispose appropriately after bleach 10% and in a biohazard labeled trash.

8. Use a box cutting blade and size 15 disposable scalpel in a 10 cm glass Petri dish.
   a. Perform the mincing in a small amount (about 3 mL) of Digestion media to enhance the mechanical digestion process. Take care to mince the muscle tissue with the box cutting blade and scalpel to as small (<1 mm^2) clear cut pieces, avoiding crushing or smashing the tissue. Proper mechanical digestion will ensure that the dissociation process will be as efficient as possible (Figure 2).
9. When thoroughly minced, transfer to a 50 mL conical tube. The more minced it is the easier to pipette or scoop out the muscle to the conical tube. Use sterile PBS to retrieve the pieces in the Petri dish and place all inside the conical tube.

Phase IIb. Micro-dissociation: enzymatic dissociation of the muscle

Timing: 2–3 h

The goal of the micro-dissociation and clean up phases is to go from clumps of cells to a clear single-cell suspension using enzymatic dissociation involving collagenase, trypsin, DNase, filters, and washes.

10. Resuspend the minced muscle in the prewarmed digestion media with collagenase, at 10 mL of media per 1 g of muscle. Example: For 5 g muscle sample, 50 mL of media will be needed. For

large samples 5 g or more we recommend having an even number of conical tubes to hold the sample and the media without overflowing the tube. In the same example, we would place the 5 g of muscle in two conical tubes each with 25 mL of digestion media with collagenase. Close the lid well, as you will place the sample in the shaker next.

11. Place on mixing platform in a 37°C incubator for 35 min, shaking gently and continuously. Use a shaker inside the incubator in the cell culture room. Every 10 min swirl and gently flick the tubes a few times during the incubation to dislodge the pellet and ease the penetration of the enzymes into the myofibers.

Note: Prior to use, the 13 gauge needle should be washed well in 70% ethanol, followed by milli Q water and then in sterile PBS; after inserting the syringe, aspirate the sterile PBS a few times to ensure cleanliness and removal of harsh chemicals prior to any contact with the tissue. The 13 gauge needle can be reused. It should be cleaned and disinfected after each use using a brush with soap and 10% bleach and kept in 70% ethanol.

12. In the 50 mL conical tube where the sample is, break up the tissue by passing through the 13 gauge needle attached to a 60 mL syringe about 10–20 times until the tissue is grossly dissociated. After that, pass the suspension through a 16-gauge needle attached to a 20 mL syringe about 10–20 times followed by a 19-gauge needle 10 times (Methods video S1).

Note: Slowly move the media with the tissue up and down the syringe. If you find clogs, gently pull them out of the eye of the needle with tweezers. Usually the clogs are connective tissue; if this is the case, you can remove them gently from the media. Be gentle to avoid any spilling of the precious sample. If the clumps are muscle, put them back on the Petri dish and mince more with the box cutting blade, then pass through the needle 10 times more. Stop soon after you get a homogeneous solution that does not clog the needle. Important to avoid generating bubbles, do not aspirate air and do not do it too many times or too fast as it may damage the cells.

13. Place the sample back in the 37°C mixing incubator for another 35 min. The sample should be in the original 50 mL conical tube with digestion collagenase buffer for a total time of 1 h 10 min. It is recommended to resuspend the pellet by aspirating with a wide-bore 1 mL tip a few times during this stage of the incubation to facilitate penetration of the collagenase all throughout the tissue.

Note: By now 3 mL of 0.25% trypsin for every gram of tissue should be warmed to 37°C for later use. As well, ensure the DNase media is ready by now and kept at RT. The DNase media should be prepared fresh the day of the digestion.

14. After 1 h and 10 min TOTAL time of tissue digestion, dilute and rinse the suspension with ice cold PBS w/o Ca++ or Mg+ by filling up the 50 mL conical tube.
15. Centrifuge the sample at 2,000 rpm (500 RCF) for 5 min at 4°C and then aspirate the supernatant.
16. Resuspend the pellet completely in residual supernatant and wash again with ice cold PBS w/o Ca++ or Mg+.

Note: Pipette slowly and with wide-bore pipette tips to increase cell viability.

17. Centrifuge the sample again at 2,000 rpm (500 RCF) for 5 min at 4°C, and then carefully aspirate the supernatant.
18. Resuspend the pellet completely in the residual liquid.

19. Add 3 mL of 0.25% trypsin for every gram of tissue (starting weight), mix gently and place in a 37°C warmer for 5–12 min according to weight of the sample. If less than 1 g, incubate for 5 min and if equal or more than 2 g incubate for 10 min to a maximum incubation time of 12 min.
20. Add 1.5 mL of FBS per gram of tissue to quench the trypsin and mix gently by swirling the tube around.
21. Fill up to top with 2% FBS in sterile PBS.
22. Centrifuge the sample at 2,000 rpm (500 RCF) for 5 min at 4°C, and then aspirate the supernatant.

⚠ CRITICAL: Only aspirate the supernatant after centrifugation at 2,000 rpm (500 RCF) for 5 min at 4°C in a refrigerated Benchtop Centrifuge. Watch the pellet at all times after centrifuging to avoid losing cells. Always place the tubes in the centrifuge in the same way, so you know where the pellet is located (Figure 4). Knowing the expected position of the pellet is especially useful when working with a very limited number of cells, as the pellet cannot be seen. Utmost care should be taken when aspirating the supernatant to not touch the pellet and leave enough supernatant after aspiration. When changing tubes, wash a few times and collect the washes in the new tube to try to collect all the cells throughout the process.

Phase IIc. Clear single-cell suspension

23. Incubate the sample with DNase media (final concentration of 10 μg/mL in DMEM). Add 3–5 mL of DNase in the conical tube to the sample and incubate 5 to 10 min at RT.

Note: This DNase step reduces cell clumping, and it is highly recommended to do.

24. Add 5 mL of 2% FBS in PBS.
25. Centrifuge the sample at 2,000 rpm (500 RCF) for 5 min at 4°C, and then aspirate the supernatant.
26. Resuspend in 5 mL of 2% FBS in PBS.
27. Place a 40 μm sterile cell strainer in a new 50 mL conical tube, label accordingly, and pipette the cell suspension through the cell strainer using a 1 mL wide-bore tip. Use 2% FBS in PBS to wash and recover most of the cells from the 50 mL conical tube and place over the filter (Figure 3). The sample should easily go through the cell strainer filter but if you find the filter clogs, try carefully centrifuging the sample for 1 min and filter again through a new sterile cell strainer.

Note: Do not skip the cell strainer filtration step, otherwise clumps may clog the MACS column or the sort later on and compromise the flow and yield of your sample.

28. Centrifuge the sample at 2,000 rpm (500 RCF) for 5 min at 4°C, and then aspirate the supernatant, leaving 0.5 mL safe margin to protect the integrity of the pellet.
29. Resuspend the pellet and perform red blood cell lysis by adding 1 mL ice cold ACK per gram of tissue (starting weight) and incubate on ice for 5–7 min. Red blood cell contamination varies with individual tissues and storage sizes. Satellite cells are remarkably resistant to ACK exposure (Figure 4).
30. Wash the sample by filling the conical with 2% FBS in PBS.
31. Centrifuge the sample at 2,000 rpm (500 RCF) for 5 min at 4°C, and then aspirate the supernatant.
32. Wash the 50 mL conical tube with ice cold MACS buffer up to the top, centrifuge, and aspirate supernatant. Resuspend the pellet in 2 mL of MACS buffer.

Phase IId. MACS and antibodies for fluorescence-activated cell sorting (FACS)

Note: The MACS columns help to eliminate non-target cells like hematopoietic cells (CD45+ and CD31+). The advantages of using the MACS columns are shorter FACS sorting time and

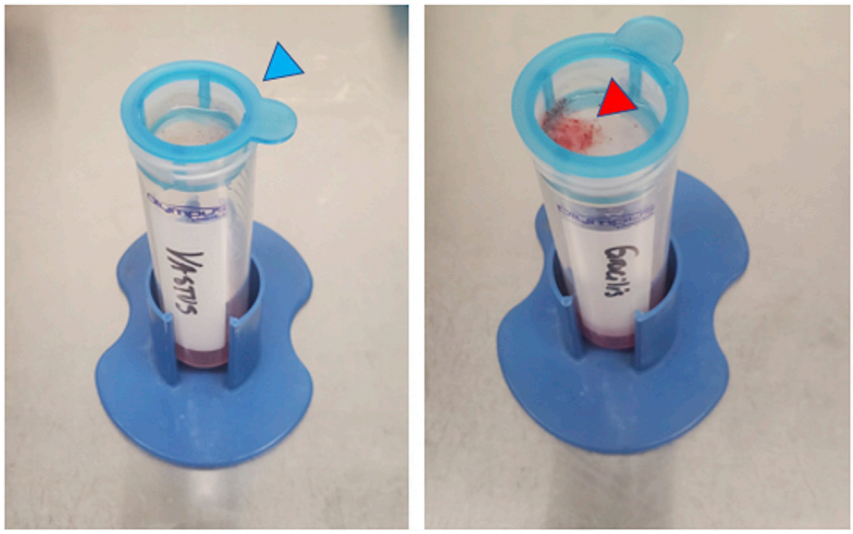

Figure 3. Ensure a clear single-cell suspension

Once the sample has been mechanically and enzymatically dissociated, it is filtered through a cell strainer 40 μm filter (blue arrow) fitted in a 50 mL conical tube. The red arrow shows the connective tissue remaining in the filter after filtration.

better visualization of the enriched populations during the sort as there is less noise signal. For experiments that require high purity of Hu-MuSC (i.e., 'omics), the MACS is recommended in order to maximize removal of contaminant cells. Nevertheless, the MACS step is optional, and it is possible to isolate Hu-MuSC without it.

33. Add 5 μL Fc blocking reagent for every gram of tissue to the conical sample tube, mix gently, and incubate for 5 min on ice.
34. Add 5 μL of anti-CD45 magnetic beads and 2 μL of anti-CD31 magnetic beads for every gram of tissue to the sample tube, mix gently, and incubate for 30 min on ice.
35. In the meantime, set up the magnetic columns as in Figure 5. The MACS LD column has to be attached to the MidiMACS separator magnet attached to a MACS MultiStand (see set up in Figure 5). Make sure the column clicks in by pushing it gently all the way toward the separator magnet. Prepare each column by adding 5 mL of MACs buffer and discard this first flow. We have been using one column for up to 3 g of muscle in order to avoid clogging the column.
36. Wash the sample by filling the 50 mL conical tube with MACs buffer.
37. Centrifuge the sample at 2,000 rpm (500 RCF) for 5 min at 4°C, and then aspirate the supernatant.
38. Resuspend the pellet completely in residual liquid -about 500 μL- and use a wide-bore pipette to release the drop of cells on top of a MACS LD column. Repeat 2 or 3 times with 500 μL of MACS buffer to wash the cells out of the 50 mL tube. Fill column with 3–5 mL of MACS buffer. Allow the sample to run through the column all the way, it may take up to 45 min.

Note: We do not use the separation columns if the sample is equal or less than 1 g because there is substantial satellite cell loss in the process, a trade-off for sorting speed. In that case, we proceed directly to the incubation with FACS antibodies with all appropriate controls for the gating and sort.

39. Collect the FLOW-through in a new 15 mL conical on ice and then elute the BOUND fraction in a separate 15 mL conical by adding 3 mL MACS buffer to the column and plunging after the removal of the column from the magnet.

Note: The FLOW sample will contain the majority of Hu-MuSC. The main advantage of using the MACS column is speeding up the sorting, since the target cells are more concentrated in the

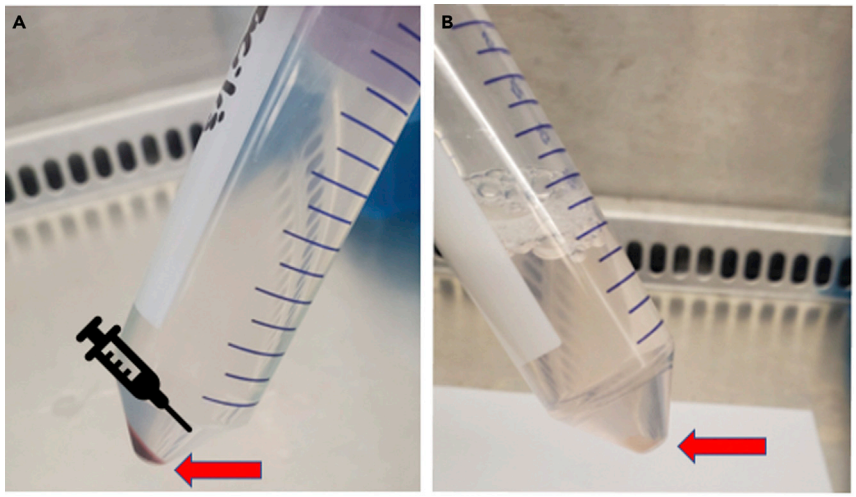

Figure 4. ACK treatment to lyse red blood cells

The red arrows show the pellet before (A) or after (B) the ACK treatment. Note the anemic pellet in (B). It is crucial to know the position of the pellet as it gets whiter and smaller. The icon syringe in (A) serves to illustrate the position of the tip of the pipette when aspirating the supernatant, distant enough from the pellet.

FLOW sample, and you should always sort this sample first. The bound still contains some Hu-MuSC you can recover on additional sorting time. For example, from the FLOW obtained processing a 5 g gracilis muscle sample from a 54-year-old female, we isolated 20,500 Hu-MuSC during a 15 min sort and from the BOUND only 500 more cells were collected in a similar amount of time.

40. Start preparing the master solution for antibodies according to ONE of the following panels:

Panel A. CXCR4 +, CD56+ and CD29+			
Antibody against	Channel/Filter	1× (µL)	5× (µL)
CD31	PAC BLUE/Violet FA	2	10
CD34	Efluor 450/Violet FA	2	10
CD45	Pac blue/Violet FA	2	10
CD56	APl770/Red A-A	8	40
CD29	FITC/Blue EA	3	15
CXCR4	PE = YG-DA	3	15

Panel B. (CXCR4 +, CD56+ and CD29+) + CAV1+ and -			
Antibody against	Channel/Filter	1× (µL)	5× (µL)
CD31	PAC BLUE/Violet FA	2	10
CD34	Efluor 450/Violet FA	2	10
CD45	Pac blue/Violet FA	2	10
CD56	APC violet/ Red A-A	8	40
CD29	FITC/ Blue EA	3	15
CXCR4 Biotin		3	15
CAV1	PE /YG-DA	1	5
Secondary Antibody			
CXCR4 Streptavidin	Unconjugated APC/ Red C-A	1:500	1:500

Figure 5. Magnetic cell separation

Setup for the magnetic cell separation (MACS LD) of hematopoietic cells (CD45+ and CD31+) from the cell suspension by means of antibodies and magnetic columns.

41. Incubate the cells with the selected panel of antibodies during 20–30 min on ice.

Note: Prepare the Controls for proper calibration and gating. The controls should include:

a. **Compensation controls**: Make single-color isotype compensation controls for each fluorochrome using beads. 1–2 drops of beads with the same amount of 1× antibody used for that specific wavelength. Shake the beads vigorously as they tend to clump together.

Note: Use new beads as old beads tend to stick to each other.

b. **Unstained cells**: Take an aliquot from the BOUND and do not add any antibody, not even cell viability marker.
c. **FMO with cells** (Fluorescence minus one): all antibodies but one. Usually done for the most critical antibody(ies). We routinely use the FMO for CXCR4 and for CAV1 in panels A and B respectively.

42. Wash the samples with MACS buffer by filling the conical tube to 15 mL.
43. Centrifuge the samples at 2,000 rpm (500 RCF) for 5 min at 4°C then aspirate the supernatant.
44. Resuspend the pellets gently in 100 μL MACS buffer.
45. Add the antibodies prepared according to panel A or B and incubate 30 min on ice, in the dark.
46. Add 2 mL MACS buffer to the 15 mL conical tube.
47. Centrifuge the samples at 2,000 rpm (500 RCF) for 5 min at 4°C, then aspirate the supernatant.
48. Resuspend in 200 μL or 400 μL (depending on how concentrated you expect your sample to be) of MACS buffer with sytox blue at a dilution of 1:1,000 and place the resuspended cells on top of the filter cap of the flow cytometry tubes.

⚠ CRITICAL: The final steps are most critical to keep small volumes safe and avoid any loss of the sample; make sure the cap of the flow cytometry tube is tightly closed before adding the sample on top to the filter cap. It should click.

49. Centrifuge the samples at 2,000 rpm (500 RCF) for 60 s min at 4°C. Keep samples at 4 degrees and sort as soon as possible. Add sytox blue again if the sort takes more than 1 h.

Note: Sort the cells into low retention 1.5 mL Eppendorf tubes to reduce the number of Hu-MuSC lost by tube wall attachment. This is especially critical when you have very limited amount of starting material.

Phase III. Fluorescence-activated cell sorting (FACS) of Hu-MuSC

Total duration of the sort: Around 2 h for proper compensation, controls and sort one sample of 1–2 g.

50. The last step should have yielded a clear cell suspension where ideally, most cells are single, (some doublets -two cells together) and no clusters of cells or debris. Cells are exposed to the antibodies with the selected fluorochromes targeting the cell membrane. The target population of Hu-MuSC will be positively sorted for CXCR4+, CD56+ and CD29+. Cells with hematopoietic markers such as CD45, CD31, and CD34 will all be discarded on the Pac blue channel.

⚠ CRITICAL: The sorter has to be professionally set up before use. Next the compensation phase will ensure the detection of each of the fluorochromes without significant overlap or appropriate compensation for it.

Phase IIIa. Compensation

51. Run single-color isotype compensation controls on the flow cytometer to determine gating. We use FACS DIVA software standard with the sorter. The following are instructions to achieve the compensation using FACS DIVA.
    a. Under Experiments, select Create Compensation.
    b. First, adjust the voltages and do not record yet.
    c. Run a single compensation control for each channel. Set the P2 on the shoulders of the positive signal per channel. Notice where positive is.
    d. Run compensation. Wait for the confirmation feedback that it is done.

Note: In the histogram used in the compensation, define the bandwidth of positive signal, tracing vertical lines over the narrow shoulders of the positive peak curve to define the range of positive fluorescence intensity for that channel and avoid or compensate for wavelength overlap with other channels.

Phase IIIb. Gating

52. Once the compensation has been completed in a satisfactory manner, it is advisable to run the controls - the unstained control followed by the FMO- and set the gates accordingly.
    a. Run and record the Unstained control.
    b. Run and record the FMO control.
    c. Run the first sample at low speed and adjust the gates.
    d. Do not change any parameter or gating after all is compensated and keep these same parameters for all the samples.

Phase IIIc. Sorting

⚠ CRITICAL: Ensure professional setup and calibration of the sorter. In the sorter window, select 4 purity mode, check the window where the sort is saved continuously for later quality control, and constantly monitor the flow rate to 1 or 2. The flow should be uniform and regular and not exceeding the recommended 7,000 events/s for the 100 μm nozzle.

Parameters in the droplet camera should be carefully monitored during the sort. Make sure the angle of the flow of sorted cells into the collection tubes is placed right at the center of the tube on the collection media, and not by the tube wall, as it compromises the viability of the cells. Having an expert support team to troubleshoot problems with the sorters during sorting is highly recommended.

Note: The size of the nozzle we use is 100 μm considering that the size of the satellite cell is 8–10 μm. Meticulous fine-tuning of the quality of the cell flow in the flow chamber and the points where the first drops detach from the stream and can be analyzed individually have to be professionally calibrated for optimal sorting. The panel of fluorescent antibodies to sort satellite cells and subpopulations have to be deliberately planned and executed.

⚠ CRITICAL: Do not vortex the cells at any point as this negatively affects cell viability.

53. Run samples on the flow cytometer and sort satellite cells; sort scheme: FSC/SSC -> viable singlets -> negative depletion of CD31/CD34/CD45/sytox positive cells -> CXCR4+ -> CD56/CD29 gate (see Figure 6).
54. Collect CD31-/CD34-/CD45-/sytox-/ CXCR4+/ CD56+/ CD29+ cells directly into the satellite cell collection media tubes.
55. During the sort, record some key monitoring parameters such as flow rate, drop 1. Be ready to take note of the number of sorted cells, as well as the percentage of the sorted population over the overall cell population, etc.
56. Keep the vials on ice after the sort and proceed immediately with experiments or cell cryopreservation.
57. Export and save all the FCS files for later quality control and record of the sort.

Phase IIId. QC of the FACS

58. Retrospective analysis of the sample is recommended after the sort using Flow Jo and the exported FCS files. An example of a sort is shown in Figure 6. Note the single-color compensation controls used to set up the calibration and additional controls such as unstained and FMO (fluorescence minus one – in this case CXCR4 PE) to accurately draw the gates to sort Hu-MuSC (CXCR4+, CD56+ and CD29+) in both the "flow" and "bound" samples.

Phase IV. Applications and preservation of Hu-MuSC

59. After the sort, it is recommended to centrifuge for 10 min at 2,000 rpm (500 RCF) 4°C to collect all the cells, aspirate the supernatant, and resuspend in 1 mL of fresh media or 0.04% BSA in PBS (for immediate use) or freezing media for cryopreservation (see below).

Note: Countess and Trypan Blue staining can be used to assess cell numbers and cell viability after the sort. This is not recommended for less than 10,000 cells as this low cell count might be out of range for an accurate quantification using the Countess instrument.

Phase IVa. QC of the sorted cells: satellite cell PAX7 expression

60. To assess if the sorted cells correspond to the expected highly purified viable human satellite cells, it is recommended to do QC tests to validate the expression of PAX7 as follows:
    a. PAX7 Immunostaining: Cells can be plated on Matrigel precoated dishes, seeded for at least 4 h at 37°C and then fixed with PFA 4%, blocked and incubated with PAX7 antibody (dilution 1:50 from the concentrated PAX7 antibody from DHSB) to verify the presence of PAX7 in the nucleus. Satellite cells are very small cells approximately 8–10 μm in diameter, and after the sort they look compact and round. Visualization under 20× to 40× light and fluorescence

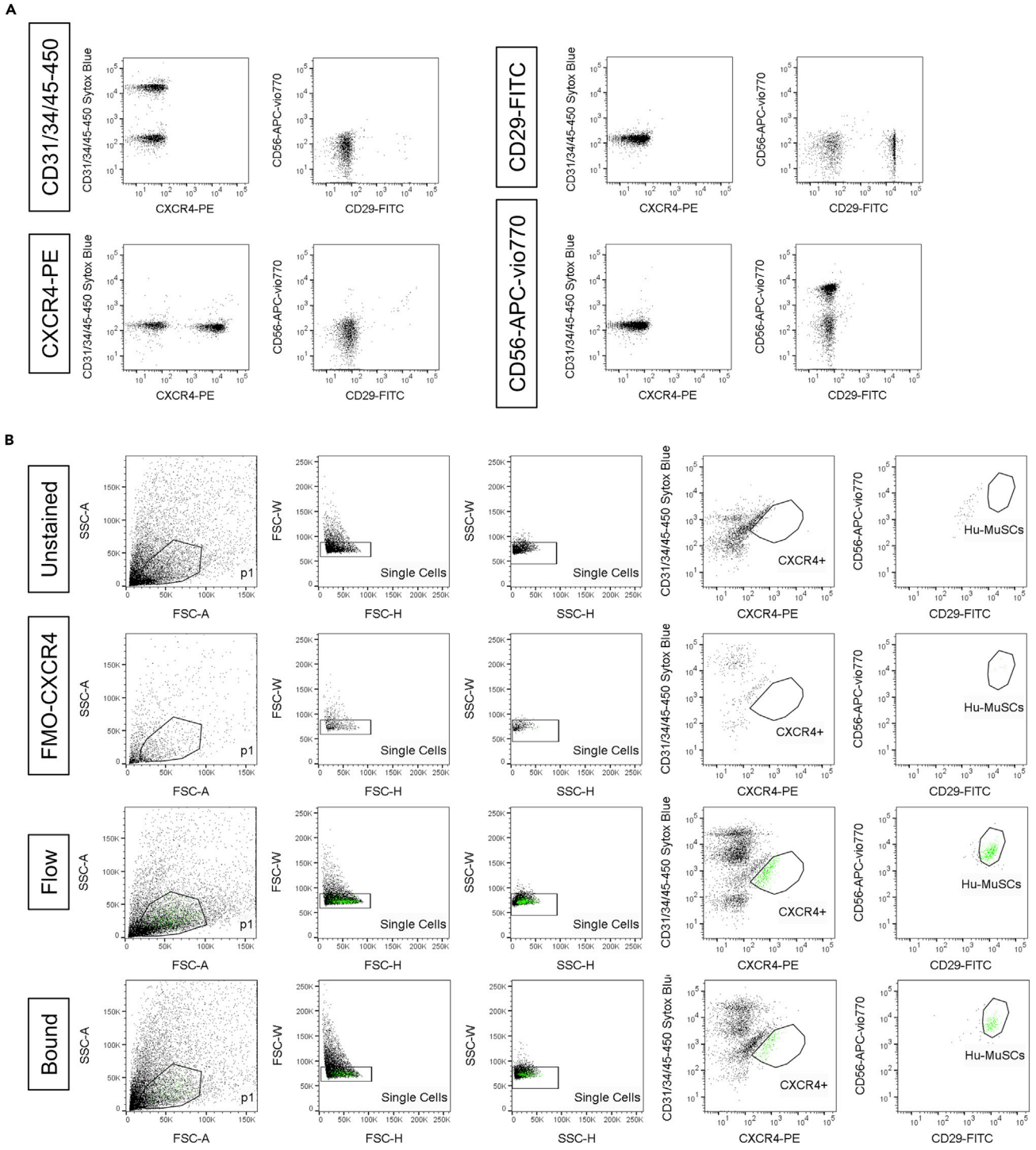

Figure 6. Representative FACS profiles for the isolation of human satellite cells

Hu-MuSC were isolated from a gracilis muscle from a 54-year-old female.

(A) Single-color compensation controls. Beads stained with each individual antibody and plotted on the 4 colors of interest.

(B) The initial population is gated for viable singlets using forward and side scatter. CXCR4 gate is defined by the all negative in the unstained and CXCR4 FMO and positive in the sample gating for CXCR4+/sytox negative cells. The last gate defines CD56+ and CD29+ in the FLOW and BOUND samples to sort for Hu-MuSC.

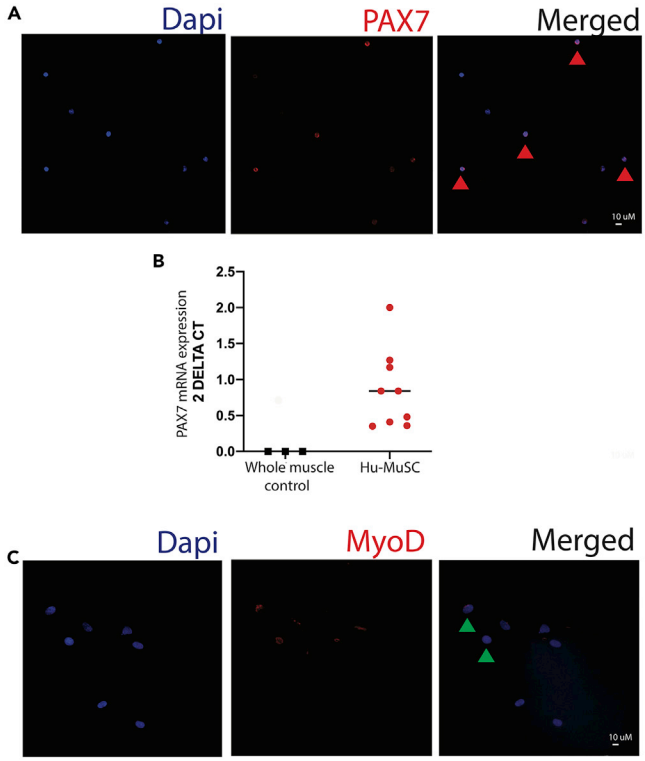

Figure 7. PAX7 and MYOD expression in sorted Hu-MuSC

Three hours after the sort, almost 100% of Hu-MuSC express PAX7 but by 12 h, this decreases to an average of 75%.

(A) Hu-MuSC derived from a vastus lateralis muscle from an 83-year-old male plated 12 h after the sort, an average 75% of SC show a positive nuclear PAX7 immunostaining in various degrees of intensity. Red arrowheads indicate Hu-MuSC that have expression of PAX7. Scale bar: 10 μm.

(B) *PAX7* mRNA expression by RT-QPRC of Hu-MuSC, right after the sort, from 5 different human donors including: temporalis from a 20-year-old female, gracilis from a 24-year-old female, extensor digitorum longus from a 63-year-old male, rectus abdominis from a 67-year-old female, tensor fascia latae from a 73-year-old male. Sorted cells from whole muscle (normally going to waste channel) were used as controls showing no *PAX7* expression.

(C) Hu-MuSC derived from a gracilis muscle from a 58-year-old female plated 3 days after the sort show positive nuclear MYOD immunostaining (green arrowheads). Scale bar: 10 μm.

microscopy is required. Immediately after the sort we are able to isolate a pure population of satellite cells, with PAX7 detectable by immunostaining in >95%. This number declines to 75% positive PAX7 cells 12 h after plating (Figure 7A).

b. *PAX7* RT Q-PCR: RNA isolation of the sorted cells can be done with Qiagen RNeasy kit protocol eluting final volume to 14 μL. Use the RNase free DNase. The extraction usually yields 50–500 ng total RNA for 5,000–10,000 Hu-MuSCs. After RNA isolation, continue immediately to cDNA transcription. It is important to do cDNA amplification up to 18 cycles. Run control samples (pre-amp and no pre-amp) in the latter qPCR to verify that the ΔCt is correctly maintained upon amplification. Proceed to reverse transcription using *PAX7* Taq or Sybergreen primers to detect and quantify *PAX7* transcript (Figure 7B).

c. Differentiation potential: The capacity of Hu-MuSC to differentiate into muscle progenitors and express muscle specific markers such as MYOD, MYH1, and MYOG can also be tested a few days after the sort. Figure 7C shows MYOD staining in Hu-MuSC plated in growth media 3 days after the sort.

Phase IVb. Applications of Hu-MuSC

Purified Hu-MuSC can subsequently utilized in experiments tailored to the users' specific interests such as phenotypic characterization, or a variety of assays.

Cell dynamics can be visualized using time-lapse video-microscopy to assess time to first division, migration, cellular mobility (Barruet et al., 2020).

Recent development in techniques to study single-cell 'omics (Barruet et al., 2020) are applicable to purified Hu-MuSC, and concentration of the desired cells using the approaches described here permit deeper analysis of single-cell data by virtue of analyzing more cells that without interference from separate cell types in a sample. For single-cell RNA, sorted Hu-MuSC were centrifuged for 10 min at 2,000 rpm (500 RCF) at 4°C, followed by resuspension in 50 μL of BSA 0.04% in PBS and immediately submitted for scRNA seq.

Xenotransplantation is another function application to test the capacity of Hu-MuSC to engraft, regenerate, and respond to *in vivo* exposures and injury (Garcia et al., 2018).

Phase IVc. Preservation of Hu-MuSC

61. To cryopreserve, Hu-MuSC are suspended in DMEM/F12 with 20% FBS, 1% Insulin-Transferrin-Selenium (ITS), 1% glutamine, 1% gentamicin, and 10% DMSO, and frozen at a cooling rate of −1°C/min overnight as follows:
    a. Centrifuge at 2,000 rpm (500 RCF) for 10 min at 4°C.
    b. Wash the cells in sterile cold PBS.
    c. Centrifuge at 2,000 rpm (500 RCF) for 4 min at 4°C.
    d. Resuspend in 1 mL of Freezing media (DMEM/F12 with 20% FBS, 1× ITS, 1× glutamine, 1× gentamicin, and 10% DMSO).
    e. Label the tube with name of the cells and sample identifiers. Record the number of cells, the date, and any other pertinent information for your experiments.
    f. Place in an RT freezing block in a −80°C freezer.
    g. For long term storage transfer to a liquid nitrogen cell bank and place in well labeled plastic boxes with appropriate holes to allow drainage. Record storage in a database.

62. Thawing Hu-MuSC after cryostorage:

    Note: We have successfully thawed and cultured Hu-MuSC that were in cryostorage for 4–5 years.
    a. Remove cryovial(s) from liquid or vapor-phase nitrogen storage and immediately thaw in the heating rocks or water bath at 37°C. Remove when a small frozen portion still remains in the vial.
    b. After thawing is complete, clean the vial with 70% alcohol and KimWipes.
    c. In a biosafety hood, gently transfer thawed cells to a 50 mL conical tube using a wide-bore pipette tip.
    d. Using a wide-bore pipette tip, rinse the cryovial with 1 mL warm complete growth medium. Add the rinse medium dropwise (1 drop per 5 s) to the 50 mL conical tube while gently shaking the tube. Dropwise addition of medium prevents osmotic lysis.
    e. Serially dilute cells with complete growth medium a total of 5 times by 1:1 volume addition with ~1 min wait between additions. Add complete growth medium at a speed of 3–5 mL/s. For example, slowly add 2 mL medium into the existing 2 mL cell suspension, gently swirl for 5 s, and leave the tube for ~1 min. This is considered the second serial dilution. Repeat 3 more times to achieve a final volume of ~32 mL.
    f. Centrifuge cells at 2,000 rpm (500 RCF) for 10 min and resuspend in desired medium.

EXPECTED OUTCOMES

This protocol works on all cranial and somite mesoderm derived human muscles tested so far. Hu-MuSC have been isolated in our laboratory across the extremes of postnatal ages and from many

types of skeletal muscle including temporalis, pectoralis, rectus abdominis, gluteus maximus, vastus lateralis, latissimus, gracilis, biceps femoris, to mention some of the most frequently sourced muscle biopsies processed in our laboratory.

The yield can be unpredictable but is often approximately 10,000 satellite cells per gram of muscle tissue. This wide range of variability is possibly due to the donor's lifestyle, exercise, diet, age, concomitant diseases, etc. (Garcia et al., 2017)

LIMITATIONS

A limitation of any protocol that physically separates satellite cells from tissue is that gene expression is altered to a degree relative to the native unperturbed state (Tsuchiya et al., 2020). Furthermore, activation of satellite cells through the FACS process has been described as changes in the stress response and metabolic pathways (Machado et al., 2017). In mice, this problem has been circumvented by fixing the animal before SC isolation and FACS (Machado et al., 2017). Although this is not generally applicable for humans, fixing a muscle sample immediately after biopsy and before cell separation is likely feasible. However, downstream experiments with living cells are obviously not amenable to such approaches, and we note that the degree of gene expression change after isolation is far lower than that of fully activated satellite cells (Barruet et al., 2020)

Because of their size, the majority of experimental biopsy samples yield on the order of 10,000 Hu-MuSC, which limits many experiments. In addition, yield can vary for unknown reasons and may relate to age, lifestyle, comorbidities, etc. We generally aim and expect to achieve yields of 10,000 Hu-MuSC per g of muscle, but yield is not currently entirely predictable. On the other hand, very large samples (more than 10 grams) are difficult to handle. It is recommended to divide large samples into batches during the macro-dissociation phase, keeping the number of batches even to ease the centrifugation steps.

Another limitation relates to the long duration of the digestion and sorting protocol. It may take 8–10 h from macro-dissociation to sorting and at that point many downstream applications may be challenging to perform immediately right after the sort. Cryopreservation is helpful in such instances.

An area of significant challenge is the short duration of PAX7 expression once the Hu-MuSC are outside their natural niche. Within hours in culture conditions cells start losing their quiescent status, elongate, differentiate into muscle progenitors and myoblasts or divide over the first week . Less than 20% of sorted Hu-MuSC will remain PAX7 positive after 6 days in culture. Therefore, efforts to understand the molecular circuitry keeping the Hu-MuSC quiescent is of paramount importance to conserve the highest potential of stemness *in vitro*.

TROUBLESHOOTING

Problem 1

Clogs during mechanical dissociation with syringes, filtering, or during the sort. The problem is wasting of precious sample due to clogs and inadequate drainage during filter steps.

Potential solution

From the first clean up, get rid of contaminants, and/or connective/fibrous tissue to prevent clogs. Do not skip any filter step and maintain good pipetting technique: slow and steady. See the video of Macro-dissociation with syringes when there is a clog that is immediately detected and discarded.

Problem 2
Do not know where to draw the gates

Potential solution
Must have all controls, especially unstained and FMO to draw the gates with confidence. Possible causes: Expired or insufficient quantity of the antibody. Sometimes there are changes in the quality of antibodies and the quantity has to be increased if the FACS signal is low.

Problem 3
Low Hu-MuSC yield

Potential solution
Avoid low yield causes such as aspiration of the pellet, vortexing the cells, loss of the cells in any of the filtration steps due to clogging. Keep the sample on ice or refrigerated at all times. Inadequate centrifugation is another cause of cell loss and it happens when the centrifugation parameters (speed, time or temperature) are not met and there might be significant number of cells lost in the supernatant after centrifugation.

Problem 4
Contamination with other cells.

Potential solution
Prevent contamination by other cells allowing optimal sorting conditions and sorting efficacy of more than 90%.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, **Jason H. Pomerantz** (Jason.pomerantz@ucsf.edu).

Materials availability
Please refer to the Key resources table for the list of all materials, which are available commercially. This study did not generate new unique reagents.

Data and code availability
Not applicable for this protocol.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100302.

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AUTHOR CONTRIBUTIONS
K.S. did the experiments, wrote and edited the manuscript, and designed the figures. E.B. commented on the manuscript and designed a figure. J.H.P. oversaw the work and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Barruet, E., Garcia, S.M., Striedinger, K., Wu, J., Lee, S., Byrnes, L., Wong, A., Xuefeng, S., Tamaki, S., Brack, A.S., et al. (2020). Functionally heterogeneous human satellite cells identified by single cell RNA sequencing. eLife *9*, https://doi.org/10.7554/eLife.51576.

Garcia, S.M., Tamaki, S., Lee, S., Wong, A., Jose, A., Dreux, J., Kouklis, G., Sbitany, H., Seth, R., Knott, P.D., et al. (2018). High-yield purification, preservation, and serial transplantation of human satellite cells. Stem Cell Reports *10*, 1160–1174.

Garcia, S.M., Tamaki, S., Xu, X., and Pomerantz, J.H. (2017). Human satellite cell isolation and xenotransplantation. Methods Mol. Biol. *1668*, 105–123.

Machado, L., Esteves de Lima, J., Fabre, O., Proux, C., Legendre, R., Szegedi, A., Varet, H., Ingerslev, L.R., Barrès, R., Relaix, F., et al. (2017). In situ fixation redefines quiescence and early activation of skeletal muscle stem cells. Cell Rep. *21*, 1982–1993.

Tsuchiya, Y., Kitajima, Y., Masumoto, H., and Ono, Y. (2020). Damaged myofiber-derived metabolic enzymes act as activators of muscle satellite cells. Stem Cell Reports *15*, 926–940.