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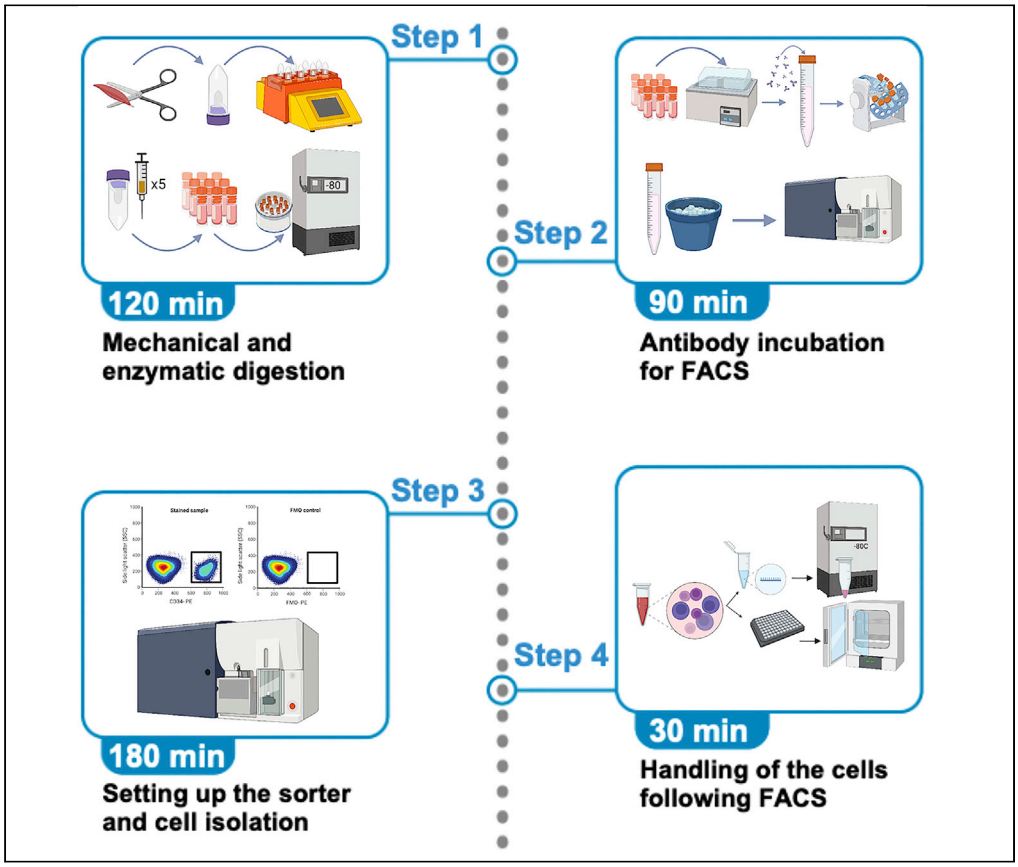
Fluorescence-activated cell sorting and phenotypic characterization of human fibro-adipogenic progenitors



The ability of stem cells to activate and differentiate is critical for maintaining the regenerative capacity of skeletal muscle. Here, we detail steps for specific quantification and isolation of primary human fibro-adipogenic progenitors and skeletal muscle stem cells using fluorescence-activated cell sorting. We describe important phenotypic traits such as time to enter the cell cycle and assessment of cell differentiation for the isolated cell populations. The technique has been applied on tissue obtained from surgery and needle biopsies.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Efficient digestion of human skeletal muscle to obtain mononuclear cells

Simultaneous sorting and quantification of stem cells

Assessment of cell activity immediately post sorting to asses stem cell function

Directions for cell culture conditions and assessment of cell differentiation

Billeskov et al., STAR Protocols 4, 102008
March 17, 2023
https://doi.org/10.1016/j.xpro.2022.102008

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Fluorescence-activated cell sorting and phenotypic characterization of human fibro-adipogenic progenitors

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https://doi.org/10.1016/j.xpro.2022.102008

SUMMARY

The ability of stem cells to activate and differentiate is critical for maintaining the regenerative capacity of skeletal muscle. Here, we detail steps for specific quantification and isolation of primary human fibro-adipogenic progenitors and skeletal muscle stem cells using fluorescence-activated cell sorting. We describe important phenotypic traits such as time to enter the cell cycle and assessment of cell differentiation for the isolated cell populations. The technique has been applied on tissue obtained from surgery and needle biopsies.
For complete details on the use and execution of this protocol, please refer to Farup et al. (2021).[1]

BEFORE YOU BEGIN

Preparation one

Consent

Always ensure approval from the local ethics committee as well as obtaining informed consent from the patients to obtain the muscle biopsies (needle as well as surgical).

Preparation two

Preparation for mechanical and enzymatical digestion of muscle tissue

Timing: 10–30 min

1. Make sure buffers are <1 month old. Prepare buffers if needed. Please see "materials and equipment" below for instructions on how to mix buffers.
2. Prepare C-tubes containing 8 mL of HAMS F10^{+} wash media (please see materials and equipment for recipe) including 700 U/mL of Collagenase II and 3.27 U/mL of Dispase II for the muscle tissue to be digested. Store on ice or at 4°C.

   Note: If the muscle biopsy is large, we recommend dividing it into several C-tubes. For optimal digestion we do not recommend more than 1 g of muscle tissue/C-tube.

3. Prepare an ice bucket as all media and tissue solution must be placed on ice when not handled during the procedures.

Preparation three

Preparation for plating of cells following FACS

Timing: 35 min

These steps coates the plates to be used for cell culture.

Note: We recommend coating the cell culture plates before the sorting of the cells to minimize the time from sorting to plating.

4. Coat the plate(s) using extra-cellular matrix coating gel (ECM) in HAMS F10^{+} wash media 1:100.
5. Place the plate(s) containing the ECM solution on a tilting table for at least 30 min at ~22°C or at 4°C.
6. Aspirate the ECM solution and replace it with growth media (please see materials and equipment for recipe) right before plating of the sorted cells. If the plates are used for experiments of proliferation using EdU the ECM solution must be replaced with HAMS F10^{+} wash media.

Note: Plates can be stored for up to one week following coating if kept at 4°C. On the day of plating of the cells aspirate the ECM solution and replace it with growth media or HAMS F10^{+} wash media for the cells to grow.

KEY RESOURCES TABLE

We have listed the reagents and equipment we have tested below. There might be other similar products from other manufactures which we have not tested.

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-human-CD45-FITC Dilution: 2 µL/(up to) 10.000.000 cells	Miltenyi Biotec	Cat# 130-114-567, RRID: AB_2726699
Anti-human-CD31-FITC 4 µL/(up to) 10.000.000 cells	Miltenyi Biotec	Cat# 130-110-668, RRID: AB_2657279
Anti-human-CD90-PE 3,6 µL/(up to) 10.000.000 cells	Thermo Fisher Scientific	Cat# 12-0909-42, RRID: AB_10670624
Anti-human-CD56-BV421 5 µL/(up to) 10.000.000 cells	BD Bioscience	Cat# 562751, RRID: AB_2732054
Anti-human-CD82-PE-vio770 10 µL/(up to) 10.000.000 cells	Miltenyi Biotec	Cat# 130-101-302, RRID: AB_2659303
Anti-mouse-CD34-APC 20 µL/(up to) 10.000.000 cells	BD Bioscience	Cat# 555824, RRID: AB_398614
Chemicals, peptides, and recombinant proteins		
Propidium Iodide (10 µL/test)	BD Bioscience	Cat# 556463
Hams F10 media incl. glutamine and bicarbonate	Sigma	Cat# N6908
Horse serum	Thermo Fisher Scientific	Cat#26050088
100× Penicillin-streptomycin (penstrep)	Thermo Fisher Scientific	Cat#15140122
Collagenase II	Worthington	Cat#46D16552
Dispase II	Roche Diagnostics	Cat#04 942 078 001
StemMACS cryopreservation buffer	Miltenyi Biotec	Cat#130-109-558
Human FcR blocking solution (20 µL/test)	Miltenyi Biotec	Cat#130-059-901
Compensation beads	Thermo Fisher Scientific	Cat#01-2222-41
Fetal Bovine serum	Thermo Fisher Scientific	Cat#16000044
Recombinant human bFGF	Sigma	Cat# F0291
DMEM (4.5 g/L glucose) incl glutamine and bicarbonate	Thermo Fisher Scientific	Cat#11965092
StemMACS™ AdipoDiff media, human	Miltenyi Biotec	Cat#130-091-677
Extra-cellular matrix coating gel	Sigma	Cat# E1270
Bio-AMF 2 growth media	Biological Industries	Cat#01-194-1A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Recombinant human Insulin	Sigma	Cat#91077C
Dexamethasone	Sigma	Cat# D4902
3-Isobutyl-1-methylxanthine (IBMX)	Sigma	Cat# I7018
Rosaglitazone	Sigma	Cat# R2408
Recombinant human TGFb	Sigma	Cat# T7039
Recombinant human PDGF-AA	Miltenyi Biotec	Cat#130-108-983
Ethanol 70%	N/A	
Paraformaldehyde 4%	Histolab	Cat# HL96753.1000
Phosphate buffered saline	N/A	
Critical commercial assays		
Click-iT™ EdU cell proliferation kit for imaging	Thermo Fisher Scientific	Cat#C10337 Cat#C10340
Software and algorithms		
ImageJ (for fluorescent image analysis)	NIH	https://imagej.nih.gov/ij/
FlowJo (10.6.1) for Fluorescence Activated Cell Sorting (FACS) and flow cytometry analysis	BD Bioscience	https://www.flowjo.com/
Other		
Petri dish for dissection	N/A	
C-tubes	Miltenyi Biotec	Cat#130-093-237
70 µm cell strainer	Miltenyi Biotec	Cat#130-110-916
30 µm cell strainer	Miltenyi Biotec	Cat#130-041-407
50 mL falcon tubes	Sarstedt	Cat#62.547.254
15 mL falcon tubes	Sarstedt	Cat#62.554.502
Cryotubes	Thermo Scientific	Cat#375418
Eppendorf tubes, 2 mL	N/A	
Pipetting tips, P1000	Sarstedt	Cat#70.3050.200
Pipetting tips, P200	Sarstedt	Cat#70.760.502
Pipetting tips, P10	vwr	Cat#613-1093
Serological pipette, 10 mL	Sarstedt	Cat#86.1254.001
20 mL syringe	Terumo	Cat#SS+20ES1
18G needle	BD Biosciences	Cat#304622
FACS collecting tubes	Falcon	Cat#352054
Gel loading tips	vwr	Cat#732-2226
RNAse free eppendrof tubes 1.5 mL	Sarstedt	Cat#72.695.400
96 well half area well plate for cell culture experiments	Corning	REF #3882
CS&T beads	BD Biosciences	655050
UltraComp eBeads	Thermo Scientific	01-2222-41
Accudrop beads	BD Biosciences	345249
Freezer, −80°C	N/A	
Freezer, −150°C	N/A	
Heated water bath	N/A	
Tuberevolver for eppendorftubes and 15 mL falcon tubes (alternatively a tilting table can be used)	N/A	
Tilting table for well plates	N/A	
Rotator (can be replaced by tilting table)	N/A	
Vortexer'	N/A	
FACS-AriaIII cell sorter (or similar cell sorter)	BD Biosciences	
Centrifuge for falcon tubes	N/A	
Centrifuge for eppendorfs	N/A	
Ventilated cell culture hood	N/A	
Finnpipette F2, P1000, P200, P10	Thermo Scientific	
Freezing container, cool cell	vwr	Cat#479-1841
gentleMACS with heaters	Miltenyi Biotec	Cat#130-096-427
Sterile forceps and surgical scissors	N/A	

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Ice bucket	N/A	
Tin foil	N/A	
Fine scale	N/A	
Liquid nitrogene (if freezing of cells for RNA analysis is performed)	N/A	
Cell culture plates (6–96 wells) of appropriate size	N/A	

MATERIALS AND EQUIPMENT

Media

The recepies for media is provided in tables below with. Here we describe the components and volumes of reagents needed to prepare wash media, growth media and the three different differentiation media for isolated MuSCs and FAPs.

Wash-buffer		
Reagent	Final concentration	Amount
Horse serum	10%	50 mL
Penstrep	1%	5 mL
HAMS F10 incl. Glutamine and bicarbonate	89%	445 mL
Total	**100%**	**500 mL**
Store media at 4°C when it is not being used. The media can be stored for one month.		

Growth media		
Reagent	Final concentration	Amount
Horse serum	10%	50 mL
Fetal bovine serum	20%	100 mL
Penstrep	1%	5 mL
DMEM 4.5 g/L glucose	69%	345 mL
Total	**100%**	**500 mL**
Store media at 4°C when it is not being used. The media can be stored for one month.		

Differentiation media for muscle stem cells		
Reagent	Final concentration	Amount
Fetal bovine serum	2.5%	12.5 mL
Penstrep	1%	5 mL
DMEM 4.5 g/L glucose	96.5%	85 mL
Total	**100%**	**500 mL**
Store media at 4°C when it is not being used. The media can be stored for one month.		

Fibrogenic differentiation media for Fibro-adipogenic progenitors		
Reagent	Final concentration	Amount
TGF-B or PDGF-AA	1 ng/mL 20 ng/mL	500 ng 10.000 ng
Fetal bovine serum	10%	50 mL
Penstrep	1%	5 mL
DMEM 4.5 g/L glucose	N/A	445 mL
Total	**100%**	**500 mL**
Store media at 4°C when it is not being used. The media can be stored for one month.		

Adipogenic differentiation media for Fibro-adipogenic progenitors		
Reagent	Final concentration	Amount
Insulin	1 μg/mL	500 μg
Dexamethasone	0.25 μM	0.25 μM
3-isobutyl-1-methylxanthine	0.5 mM	0.5 mM
Rosaglitazone	5 μM	5 μM
Fetal bovine serum	20%	100 mL
Penstrep	1%	5 mL
DMEM 4.5 g/L glucose	N/A	400 mL
Total	**100%**	**500 mL**

Store media at 4°C when it is not being used. The media can be stored for one month.

Note: Always prepare, open, and handle all media in a sterilized ventilated tissue culture hood to avoid contamination of the media.

Alternatives: For adipogenic differentiation media for fibro-adipogenic progenitors (FAPs) one may also use complete adipogenic differentiation media (see key resources table). The media can be kept at −20°C until use. Store at 4°C for up to one month following thawing of the media.

STEP-BY-STEP METHOD DETAILS

Mechanical end enzymatical digestion of muscle tissue

Timing: 2 h

These steps release the mononucleated cells from the dense skeletal muscle tissue.

Note: All media and tissue suspension must be placed on ice when not handled.

⚠ CRITICAL: All procedures must be performed in a ventilated sterile tissue culture hood to avoid contamination of the sample and for the safety of the investigator.

1. Place the muscle tissue sample in a petri dish.
   a. Make sure to keep the muscle tissue moist by adding 2–3 mL of HAMS $F10^{+}$ wash media if needed.

Note: If the muscle tissue is obtained by Bergströms needle, the tissue may be placed directly in the C-tubes without dissection (skip steps 2 and 3), as the Bergströms needle usually provides small tissue samples of 30–300 mg.[2]

2. Mince the muscle tissue using sterile surgical scissors to obtain a slurry substance (Figure 1).

⚠ CRITICAL: It is important remove all visible connective tissue and fat by using sterile forceps and scissors to be able to mince the tissue well. This leaves a greater surface area for

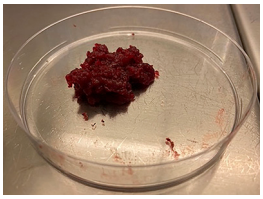

Figure 1. The muscle tissue has been minced using sterile surgical scissors

the enzymes to digest. Although the gentleMACS also mince the tissue, the initial manual mincing increases the effectiveness of the digestion and ultimately the cell yield. Optimal enzymatic digestion is of key importance for great yield of cells.[3] Please see Figure 1 for example of minced muscle tissue with all connective tissue removed.

3. Weigh the muscle tissue if evaluation of cells/mg of tissue is of relevance.
4. Transfer the minced muscle tissue to the C-tube(s). One C-tube holds up to 1 g of muscle tissue with the present set-up.
5. Place the C-tube(s) on the gentleMACS dissociator.
   a. Add heaters.
6. Choose a muscle digestion program.

Note: We use the program 37_mr_SMDK1 (1 h 1 min), which provides sufficient time for effective dissociation.[1]

7. **Pause Point:** 61 min.

Make sure to check on the gentleMACS dissociator every 15 min to ensure no clogging of the rotating blades has occurred (Figure 2).

Troubleshooting.

Please see troubleshooting problem one below on actions to perform if clogging occurs.

8. Add 8 mL of 4°C HAMS $F10^+$ wash media to each C-tube to stop the enzymatic digestion by dilution.
9. Place a 70 μm cell strainer on a 50 mL falcon tube.
10. Wash the filter using 2 mL of HAMS $F10^+$ wash media to remove the surface tension.
11. Aspirate the digested muscle suspension in and out of a 20 mL syringe using a 18 G needle.
    a. Make sure to eject to the wall of the tube to be gentle on the cells and avoid foaming.
    b. Repeat five times to ensure single cell suspension.

⚠ CRITICAL: in order to obtain single cell suspension, it is important to aspirate *all* of the muscle suspension five times.

Troubleshooting.

Please see troubleshooting problem two below on actions to perform if clogging of the needle occurs.

12. Aspirate the entire suspension into the syringe and remove the needle.
13. Eject the suspension onto the 70 μm cell strainer on a 50 mL falcon tube to remove debris by gravity.

Troubleshooting.

Figure 2. Digested human skeletal muscle
The muscle tissue has been digested on the GentleMacs dissociator and 8 mL of HAMS $F10^+$ wash media has been added. The digested muscle tissue is now a liquid substance ready to filter.

Please see troubleshooting problem three below on actions to perform if clogging of the filter occurs.

Note: If the muscle biopsy is large and divided into several C-tubes the same syringe and falcon tube can be used for those C-tubes. Remember to change the needle and the cell strainer as these may clog. One 50 mL falcon tube holds ~2 C-tubes.

14. Wash the empty C-tube(s) twice using 5–10 mL of HAMS $F10^{+}$ wash media.
    a. Transfer the wash media to the 70 μm cell strainer(s) to ensure that all cells are collected from the C-tube(s) and the cell strainer(s).
    b. Make sure to collect any remaining liquid hanging underneath the cell strainer by using a pipette in order to maximize cell yield.
15. Centrifuge at 800 × *g* for 5 min at 4°C.
16. Aspirate the supernatant.

Note: Be careful not to disturb the pellet as this will loosen. The pellet loosens with time and thus it is important to do the aspiration immediately after centrifugation.

17. If Fluorescence Activated Cell Sorting (FACS) is performed immediately, move to step 21 from here. If the cells need to be stored before FACS continue with step 18.
18. Add 1 mL of StemMACS cryopreservation buffer (or similar freezing buffer) to the pellet(s).
    a. Resuspend the cells by gently pipetting up and down 2–4 times.

Note: We recommend resuspending the pellet(s) by using a 1,000 μL pipette or larger.

19. Transfer the solution to cryotube(s) using a pipette.
20. Place the cryotube(s) in a freezing container and store at −80°C until further use.

Note: Once freezing has occurred (~ 12–24 h) the cells can be moved from the freezing container to storage in freezing boxes made of e.g., cardboard or plastic. However, we recommend moving the cells to a −150°C freezer after 24 h for preservation of cells. The cells can be stored at −80°C if -150 freezer is not available. If the cells are to be used for cell culture we have experienced the highest viability and best overall cell performance if the cells are sorted within 2–4 weeks after freezing.

Preparation of the sample(s) for sorting and samples to set up the sorter

This section includes:

Antibody incubation of the sample(s) to be sorted using FACS (steps 21–52).

Fluorescence minus one (FMO) controls (steps 52–60).

Compensation samples – propidium iodide compensation sample (steps 61–64).

Compensation samples – single-colored beads for FACS (steps 65–73).

An unstained control sample (step 32).

The section of antibody incubation of the sample(s) to be sorted using FACS is for preparation of the sample(s) to be sorted. The sections of *FMO controls, compensation samples and the unstained control* are for preparation of samples to be used for setting up the sorter.

Note: Antibody incubation can be performed on freshly digested single cell solutions or on single cell solutions, which has previously been digested and stored at −80°C or −150°C. As antibody incubation of the sample(s) to be sorted is the most time-consuming step, we recommend starting with this preparation, which is why we describe these preparation steps first. We recommend that the FMO controls (steps 52–60) are prepared alongside the sample(s) to be sorted using FACS, alternatively at the pause point in this section (step 41). Compensation samples (step 65-73) can be prepared at the pause point of the antibody incubation of the sample(s) to be sorted using FACS (step 41). When moving to the sorter, the FMO controls, the compensation samples and the unstained control sample must be analyzed on the cell sorter *before* running the full samples to be sorted using FACS, as described under the section: Setting up the cell sorter and sorting of samples (steps 74–89).

Note: In this protocol we have not included the utilization of isotype controls. This decision is based on 1) that this protocol is only intended to sort positive from negative (bimodal) cell populations; 2) that we have not included sorting of cells expressing Fc-reptors (e.g., myeloid cells) and; 3) the difficulty in obtaining isotype controls for all antibodies fulfilling the criteria needed to serve as a usefull control.[4] To confirm specificity of the sorted cells, we highly recommend to perform control stainings of freshly sorted cells to confirm uniform expression of specific cell markers (e.g. Pax7 for MuSCs and PDGFRα for FAPs).

Antibody incubation of the sample(s) to be sorted using FACS

Timing: 1 h 30 min (for step 21)

These steps allows for antibodies to react with cell surface proteins for the FACS to distinguish and sort the cells. This is to be performed on single cell solutions from digested muscle tissue.

21. Add 10 mL of HAMS F10$^+$ wash media (stored at 4°C) to a 15 mL falcon tube. Prepare one 15 mL falcon tube per sample being sorted.

Note: If antibody incubation is performed on single cell solutions from freshly digested muscle tissue which has not been frozen, move directly to step 28.

22. Thaw the digested muscle sample(s) quickly in 37°C water bath until only a small amount of ice is left in the tube.
23. Wipe down the tube(s) using 70% ethanol and place it at the sterile tissue culture hood.
24. Transfer the thawed or fresh sample(s) to the 15 mL falcon tube(s) containing 10 mL of HAMS F10$^+$ wash media.
25. Wash the tube(s) which contained the cells using 1 mL of HAMS F10$^+$ wash media from the 15 mL falcon tube(s) to collect all the cells.
    a. Transfer the wash media to the 15 mL falcon tube(s).
26. Centrifuge at 800 × *g* for 5 min at 4°C.
27. Aspirate the supernatant to remove the StemMACS cryopreservation buffer (or chosen alternative freezing buffer).

Note: Be careful not to disturb the pellet as this will loosen. The pellet loosens with time and thus it is important to do the aspiration immediately after centrifugation. While it is important to remove the HAMS F10$^+$ containing the cryopreservation buffer the dilution with HAMS F10$^+$ will also ensure this.

28. Add 200–400 μL of HAMS F10$^+$ wash media to the 15 mL falcon tube(s) to reach a final volume of approximately 500 μL.
29. Resuspend the pellet(s) by gently pipetting up and down 2–4 times.

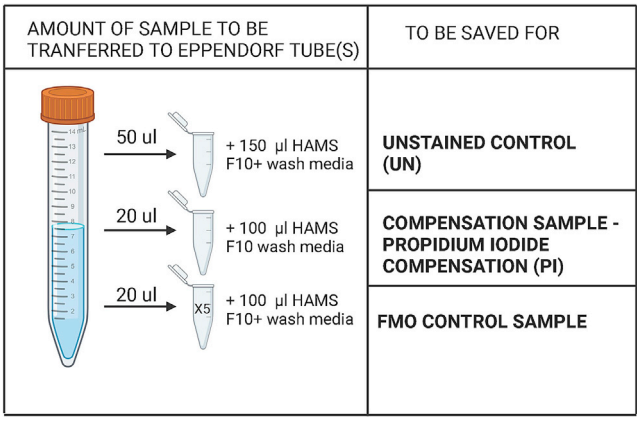

Figure 3. Graphical illustration of small samples to be saved in Eppendrof tubes

Note: The steps below (steps 30–38) are for saving small samples from the sample in the 15 mL falcon tube(s) before the antibodies are added (see Figure 3). These are to be used later for making FMO controls, compensation sample-propidium iodide compensation sample and unstained control (point 52–73).

30. Transfer 50 µL sample from each 15 mL falcon tube to individual 2 mL Eppendorf tube(s) using a pipette.
31. Place the Eppendorf tube(s) on ice and add additional 150 µL of HAMS $F10^{+}$ wash media to each Eppendorf tube.
32. Save the tube(s) on ice for later use as unstained control sample (steps 79 and 86).

Note: the non-stained control must be filtered through a 30 µm cell strainer alongside the filtering of the full-stained samples at step 47.

33. Transfer 20 µL sample from each 15 mL falcon tube to individual 2 mL Eppendorf tube(s) using a pipette.
34. Place the Eppendorf tube(s) on ice and add additional 100 µL HAMS $F10^{+}$ wash media to each tube.
35. Save the tube(s) on ice for later use to generate the compensation sample – propidium iodide compensation sample (steps 61–64).
36. Transfer 20 µL sample from each 15 mL falcon tube to 5 individual 2 mL Eppendorf tubes using a pipette (Figure 4).
37. Place the Eppendorf tubes on ice and add additional 100 µL HAMS $F10^{+}$ wash media to each.
38. Save the tubes on ice for later use as FMO controls (steps 52–60).
39. Add antibodies to the sample left in the 15 mL falcon tube(s) (see Table 1 below).

Note: The volume of antibodies are in line with the recommendations from the manufacturer as we usually sort <10.000.000 cells/sample. The volume of antibodies may need to be increase if > 10.000.000 cells are expected.

40. Place the sample(s) on a rotator or a tilting table at 4°C in the dark for 30–40 min.

Note: Wrap the tube(s) in tinfoil to prevent light from reaching the samples.

41. **Pause Point:** 30–40 min.

In the meantime, the compensation samples (steps 61–73) can be prepared.

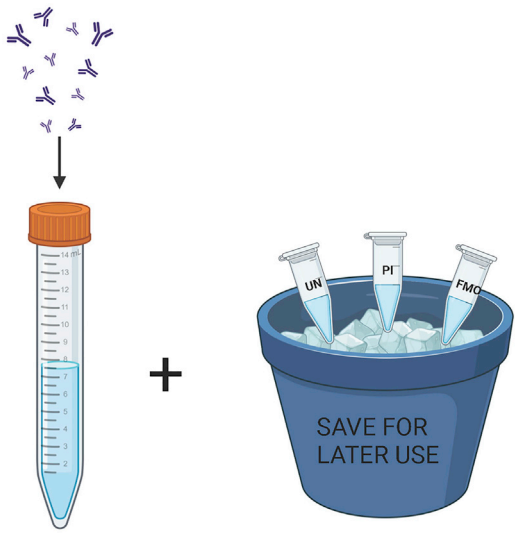

Figure 4. Full sample staining in 15 mL falcon tube while saving the samples in the Eppendorf tubes later usage

42. Add 10 mL of HAMS F10^{+} wash media to the 15 mL falcon tube(s).
43. Centrifuge at 800 × *g* for 5 min at 4°C.
44. Carefully aspirate the supernatant.

Note: Be careful not to disturb the pellet as this will loosen. The pellet loosens with time and thus it is important to do the aspiration immediately after centrifugation.

45. Add 500 μL of HAMS F10^{+} wash media to the 15 mL falcon tube(s).
    a. Resuspend the cell pellet by pipetting up and down 2–4 times.

Note: The volume of HAMS F10^{+} wash media to be used depends on the size of the biopsy. For small biopsies obtained by Bergströms needle use 250 μL of HAMS F10^{+} wash media. For larger biopsies of up to 1 g up to 1–2 mL of HAMS F10^{+} wash media must be added.

46. Place a 30 μm cell strainer on a 5 mL FACS tube.

Note: One 30 μm cell strainer must be placed on one μL 5 mL FACS tube per sample being handled as well as one for the non-stained control.

47. Transfer the solution(s) to the cell strainer(s) by using a pipette. This removes debris and aggregates by gravity.
    a. Remember to also filter the non-stained control prepared in step 32.
    b. Make sure to collect any remaining liquid hanging underneath the cell strainer(s) using a pipette.

Table 1. Antibodies for full stained sample

Antibody	Flourocrome	μL
CD45	FITC	2
CD 31	FITC	4
CD 56	Brilliant violet	5
CD 90	PE	3.6
CD 82	PE-Vio770™	10
CD 34	APC	20

Antibody panal and relevant volumes for full stained sample.

48. Wash the 15 mL falcon tube(s) using 500 μL of HAMS F10$^+$ wash media twice (the amount of media can be scaled depending on the amount of tissue in the sample as described above).
49. Transfer the 2 × 500 μL of HAMS F10$^+$ wash media to the cell strainer.
50. Prepare collection tubes for the FACS by adding HAMS F10$^+$ wash media to the collection tubes (500 μL if using FACS tubes, 100 μL if using Eppendorf tubes).
    a. Mark the tubes with ID and cell type.
    b. Place the tubes on ice.

Note: For highest yield and viability of cells move directly to the sorter.

51. Add Propidium iodide to the cells *immediately before sorting* to distinguish viable from non-viable cells.

Fluorescence minus one (FMO) controls

Timing: 1 h 30 min

These steps creates samples to be used to set up the gates for the first sort. Once the FMO samples have been run and gates have been set, the settings can be saved in the protocol on the FACS sorter for future use. Antibody incubation can be performed on single cell solutions from freshly digested muscle tissue or on solutions stored at −80°C or −150°C.

Note: All procedures must be performed in ventilated sterile tissue culture hood to avoid contamination of the sample and for protection of the investigator.

52. Take the 120 μL samples put aside for FMO controls in steps 36–38.
53. Add antibodies to the Eppendorf tubes (please see Table 2 below).

⚠ CRITICAL: As these are FMO controls remember to leave out one fluorochrome/antibody when adding the antibodies to the samples. Mark which antibody is left out on the tube.

Note: CD45 and CD31 can be made in the same tube as both are FITC-conjugated antibodies.

Note: The volume of antibodies are in line with the recommendations from the manufacturer as we usually sort <10.000.000 cells/sample. The volume of antibodies may need to be increase if > 10.000.000 cells are expected.

54. Place the Eppendorf tubes on a rotator or a tilting table at 4°C in the dark for 30–40 min.

Note: Wrap the tubes in tinfoil to prevent light from reaching the samples.

55. Pause Point: 30–40 min.
56. Add 1.5 mL of HAMS F10$^+$ wash media to the Eppendorf tubes.
57. Centrifuge at 800 × *g* for 5 min at 4°C.
58. Carefully aspirate the supernatant.

Note: Be careful not to disturb the pellet as this will loosen. The pellet loosens with time and thus it is important to do the aspiration immediately after centrifugation.

59. Add 250 μL of HAMS F10$^+$ wash media to the Eppendorf tubes.
    a. Resuspend the cell pellets by pipetting up and down a 2–4 times.
60. Add Propidium iodide to the cells *immediately before sorting* to distinguish viable from non-viable cells (do not add PI to the PI FMO sample).

Table 2. Fluorescence minus one controls (FMO)

FMO sample	Flourochrome	µL of antibodies to be added
CD45/CD31	FITC	5 µL CD56 (Brilliant Violet) 3.6 µL CD90 (PE) 10 µL CD82 (PE- Vio770™) 20 µL CD34 (APC) 10 µL Propidium iodide (PI)
CD56	Brilliant violet	2 µL CD45 (FITC) 4 µL CD31 (FITC) 3.6 µL CD90 (PE) 10 µL CD82 (PE- Vio770™) 20 µL CD34 (APC) 10 µL Propidium iodide (PI)
CD90	PE	2 µL CD45 (FITC) 4 µL CD31 (FITC) 5 µL CD56 (Brilliant Violet) 10 µL CD82 (PE- Vio770™) 20 µL CD34 (APC) 10 µL Propidium iodide (PI)
CD82	PE-Vio770™	2 µL CD45 (FITC) 4 µL CD31 (FITC) 5 µL CD56 (Brilliant Violet) 3.6 µL CD90 (PE) 20 µL CD34 (APC) 10 µL Propidium iodide (PI)
CD34	APC	2 µL CD45 (FITC) 4 µL CD31 (FITC) 5 µL CD56 (Brilliant Violet) 3.6 µL CD90 (PE) 10 µL CD82 (PE- Vio770™) 10 µL Propidium iodide (PI)
Propidium iodide (PI)		2 µL CD45 (FITC) 4 µL CD31 (FITC) 5 µL CD56 (Brilliant Violet) 3.6 µL CD90 (PE) 10 µL CD82 (PE- Vio770™) 20 µL CD34 (APC)

Description of antibodies neded for generation of FMO controls for the cell sorting panal.

Compensation samples—Propidium iodide compensation sample

Timing: 5 min (for step 61)

These steps creates a PI compensation sample which is used for generating a compensation matrix in combination with the beads prepared below.

61. Heat the Eppendorf tube containing 120 µL of unstained sample (from step 32) at 60°C–70°C for 1 min.
62. Place the tube on ice immediately (to increase with number of cells with a permeable membrane).
63. Mark the tube PI control.
64. Add 10 µL Propidium iodide *immediately before sorting*.

Compensation samples—Single-colored beads for FACS

Timing: 25 min (for step 65)

These steps creates samples which are to be used for generating a compensation matrix in the sorter software to account for spectaral overlap between fluorochromes. All tubes should be marked with antibody and fluorochrome.

65. Add One drop of UltraComp eBeads to each of the five Eppendorf tubes.
66. Add one antibody (two for the FITC conjungated CD45 and CD31 antibodies) (see Table 3 below).

Note: Add only one antibody to each Eppendorf tube containing UltraComp eBeads. CD45 and CD31 can be made in the same tube as both are conjugated to FITC.

67. Vortex the samples and incubate for 15 min at ~22°C in dark.
68. Add 1 mL of PBS to each Eppendorf tube to wash away residual anti-body.
69. Centrifuge at 800 g for 5 min at 4C°.

⚠ CRITICAL: It is very important to know where the pellet is located as the pellet is not visible. Place all the Eppendorf tubes facing the same direction (e.g., with the opening of the lid facing upwards) so that the pellets are located on the same spot in all tubes.

70. Aspirate the supernatant.

⚠ CRITICAL: It is important to not hit the area of the pellet with the pipette tip. It can be helpful to add a gel loading tip, which has a narrow opening and holds a low volume, on top of the pipetting tip.

71. Add 300 μL of PBS to each tube to wash the side of the tube where the pellet is located.
72. Pipette up and down 2–4 times to resuspend the pellet.
73. Vortex.

Note: Wrap the tubes in tinfoil to prevent light from reaching the samples.

Setting up the cell sorter—General setting of the cell sorter

These are the general steps for setting up the sorter for cell sorting.

Note: Timing depends on the number of samples to be sorted as well as the size of the samples being sorted (this may vary depending on the type of sorter)

74. Set up the cell sorter with a 100 μm nozzle and 20 psi.
    a. Allow the system to stabilize.
75. Run CS&T beads to quality control the instrument.
    a. Adjust the stream by changing the amplitude and frequency. Make sure nice uniform drops are forming.
76. Adjust the drop delay by running Accudrop beads until deflection of 97%–100% of the Accudrop beads is achieved.
77. Adjust the side streams by applying a suitable voltage for the drops to fall into the collection tubes.

Table 3. Antibodies for compensation samples

Compensation sample	Antibody	Fluorocrom	μL
FITC	CD45 CD31	FITC FITC	2 4
Brilliant violet 421	CD56	Brilliant violet 421	5
PE	CD 90	PE	3.6
APC	CD34	APC	20
PE-Vio770	CD82	PE-Vio770(TM)	10

Antibodies and volumes needed for generation of the compensation matrix using compensation beads.

a. Make sure the droplets fall into the middle of the collection tubes by adjusting the electrical charge on the side streams.

78. Set up an experiment using the software.

Note: When setting the sort layout we use "purity" for precision sort mask.

79. Run some of the non-stained sample (prepared in step step 32) for setting PMT voltages.
    a. Save at least 10 μL of the non-stained sample for step 86.
80. Run a 5–10 μL of the full stained sample (prepared in step steps 21–54).
    a. Make sure no fluorochromes are off scale.
81. Initiate the compensation setup in the FACS software.
    a. Run the compensation sample – single colored beads for FACS (prepared in steps 65–73) and the compensation sample – propidium iodide compensation (prepared in steps 61–64) following the steps designated in the software.

Setting up the cell sorter for sorting of human skeletal muscle samples

These are the steps for setting up the sorter for sorting of human MuSCs and FAPs.

Timing depends on the number of samples to be sorted as well as the size of the samples being sorted

82. Set up the gating strategy (Please see Figures 5, 6, 7, 8, and 9 below):
83. On the FSC-A and SCC plot create a gate to collect the cells of the right size and to exclude debris.
84. On the FSC-A and FSC-H plot create a gate to collect single cells only.
85. Create gates to collect skeletal muscle stem cells (MuSCs) ($CD34^{-}CD56^{+}CD82^{+}CD45^{-}CD31^{-}$) and FAPs ($CD34^{+}CD56^{-}CD45^{-}CD31^{-}$).

Note: the FAP population can be further subdivided into $CD34^{+}CD90^{-}$ and $CD34^{+}CD90^{+}$.[1]

86. Run the unstained control(s) with compensation to validate that the sorting gates are placed correctly.
87. Run the FMO controls (prepared in steps 52–60) with compensation to validate that the sorting gates are placed correctly.
    a. Make sure that background fluorescence is low in all channels and that voltages are set correctly.
88. Add 10 μL of PI to the sample being sorted *immediately before sorting*.
89. Run the sample (for expected percentage of each cell population to fall within the gates please see Table 4 below).

Note: The ideal flowrate depends on the sample (debris, single cell suspension etc.). Once the sample is running, we recommend sorting 2,000–5,000 events/second at flowrate 3–6 and never more than 8.000 events/second. It may be necessary to make small adjustments to the gates upon loading of the human sample. Adjust the gates for cell populations to always be in the center of the gates (see Table 4 for expected ranges of cell populations).

Handling of cells following FACS

Following sorting, cells can be frozen for later RNA isolation or they can be plated on ECM coated plates (please see 'before you begin' for ECM coating). The cells can also be used for other experiments e.g., protein extraction or chip analysis, which will not be described in this protocol.

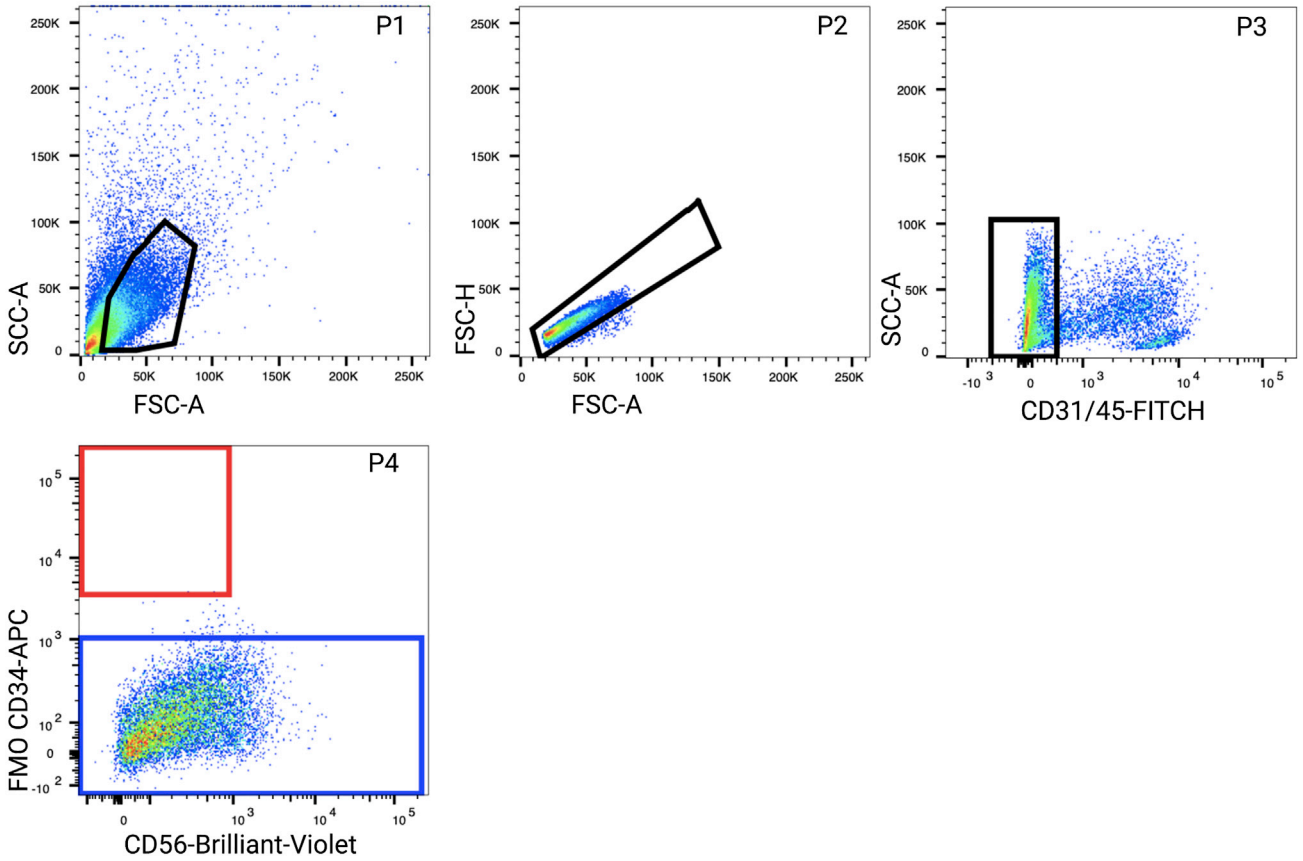

Figure 5. Representative FACS plots for FMO control for CD34-APC

(P1) FSC-A vs SCC-A plot to collect cells of the right size.

(P2) FSC-A vs. FSC-H plot to collect single cells only.

(P3) CD31/45-FITCH vs. SSC-A plot to exclude endothelial cells and immune cells through negative selection.

(P4) CD56-Brilliant-Violet vs. CD34-APC plot to distinguish 34^+ and 34^- cells. As this is the FMO CD34-APC there are no $CD34^+$ cells (red box).

Freezing of cells for later RNA analysis

Timing: 10 min

Note: The cells do not need to be handled in a sterile cell culture tissue hood.

90. Gently vortex the sample(s).
91. Transfer the solution(s) containing the sorted cells to RNAse free Eppendorf tubes.
    a. Mark with ID and cell type.
92. Centrifuge at 1,000 × *g* for 5 min at 4°C.
93. Aspirate the supernatant.

⚠ CRITICAL: It is very important to know where the pellet is located as it cannot be seen. Place all the Eppendorf tubes facing the same direction (e.g., with the opening of the lid facing upwards) so that all pellets are located in the same spot in all tubes. It is important to not hit the area of the pellet with the pipette tip. It can be helpful to add a gel loading tip on top of the pipetting tip.

94. Store the tube(s) at −80°C until analysis.

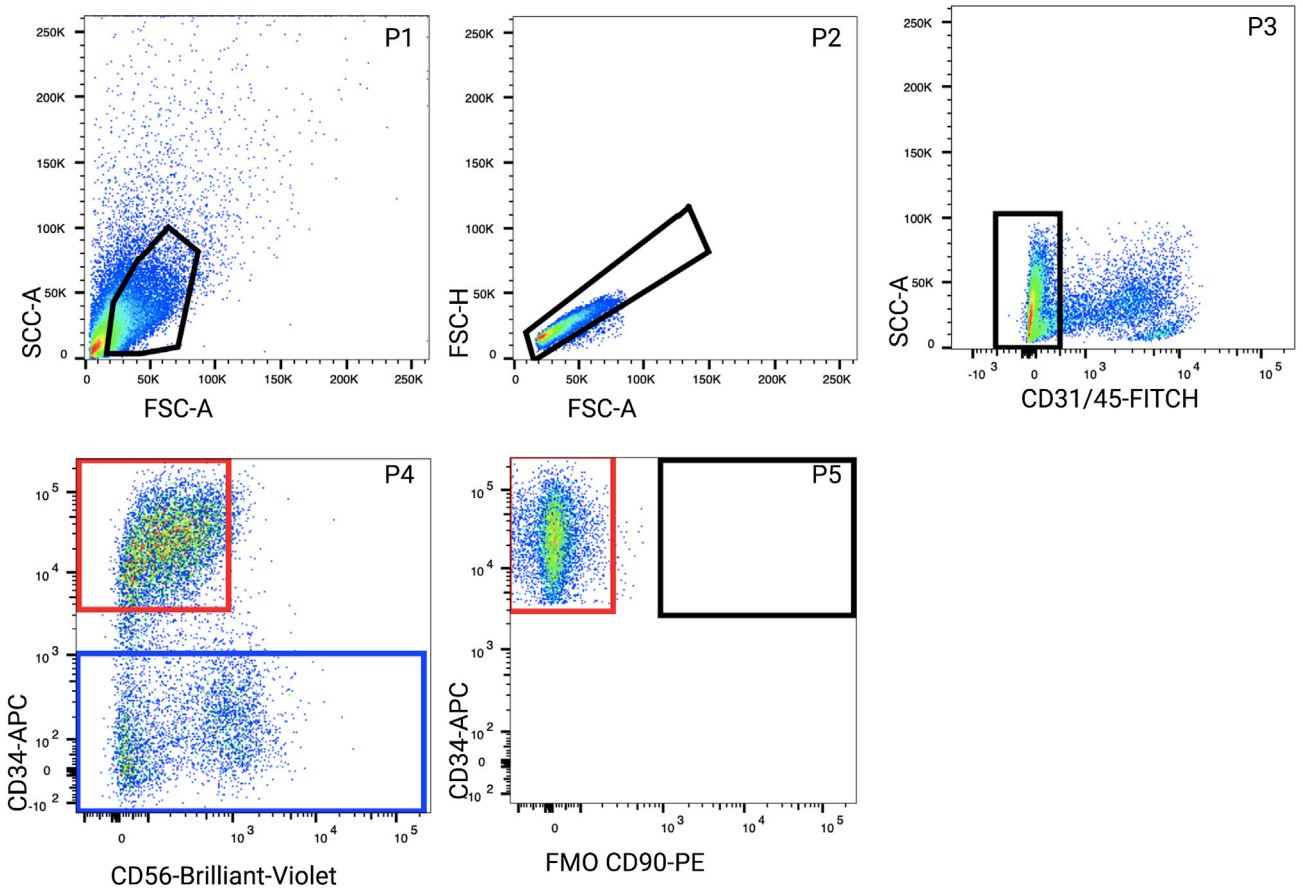

Figure 6. Representative FACS plots for FMO control for CD90-PE

(P1) FSC-A vs SCC-A plot to collect cells of the right size.

(P2) FSC-A vs. FSC-H plot to collect single cells only.

(P3) CD31/45-FITCH vs. SSC-A plot to exclude endothelial cells and immune cells through negative selection.

(P4) CD56-Brilliant-Violet vs. CD34-APC plot to distinguish 34^+ cells (red box) and 34^- cells (blue box).

(P5) FMO CD90-PE vs. CD34-APC plot to distinguish between the $CD90^+$ and $CD90^-$ populations of the 34^+ cells from (P4). As this is the FMO CD90-PE control there are no $CD90^+$ cells (black box).

Note: The samples can be snap frozen in liquid nitrogen before storage.

Plating of MuSCs and FAPs for cell culture experiments of proliferation and differentiation

Timing: 10 min depending on the number of samples to be plated

95. Plate the cells at density of approximately $1 \times 10^4/cm^2$.
    a. Add growth media at an appropriate amount for the well size choosen.

Note: If experiments of proliferation using EdU are to be performed (steps 97–102) plate the cells in HAMS F10+ wash media instead of growth media.

96. Place the cell culture tray(s) in a 37°C 5% CO_2 incubator.

⚠ CRITICAL: Change the media every other day for the cells to always be supplied with nutrients and stable pH.

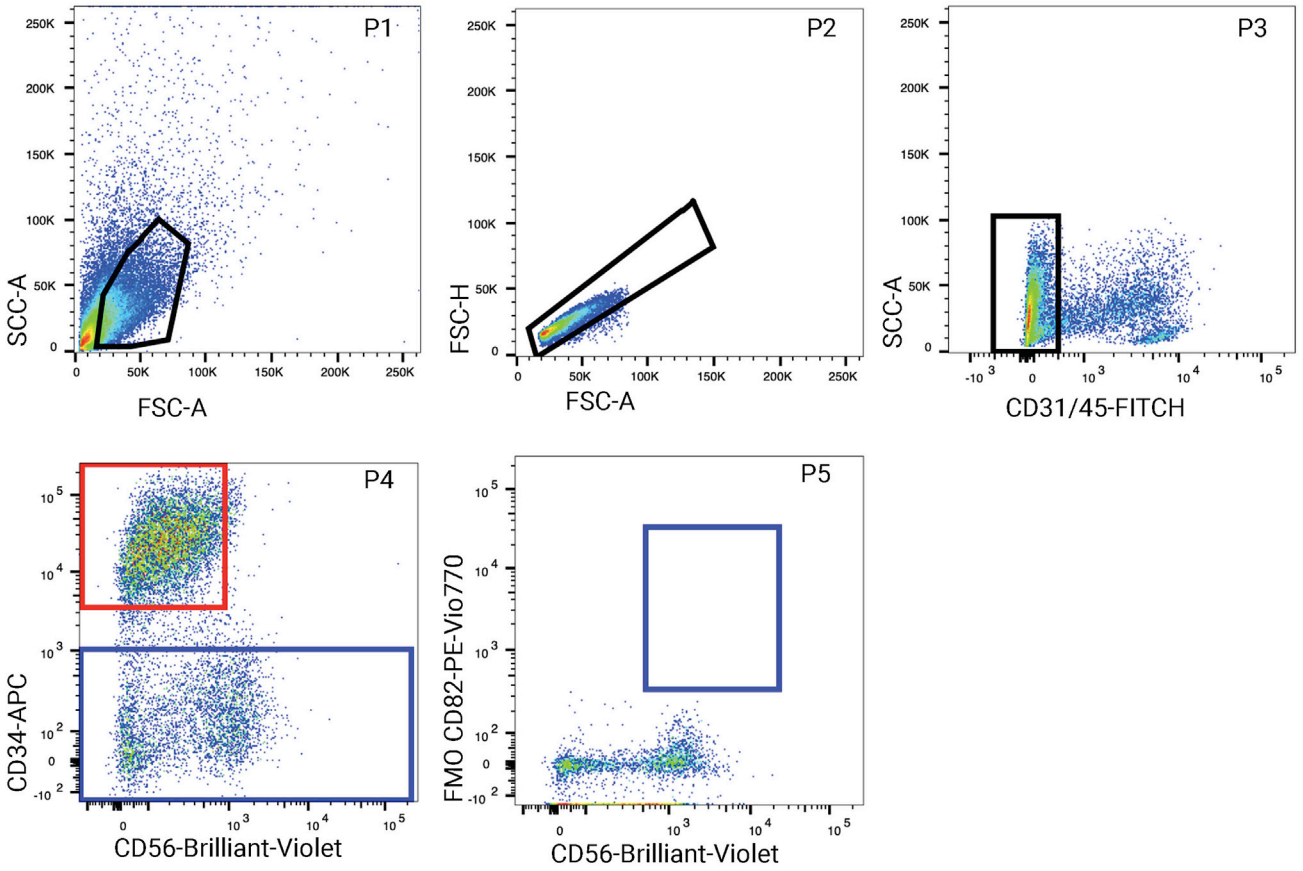

Figure 7. Representative FACS plots for FMO control for CD82-PE-Vio770

(P1) FSC-A vs SCC-A plot to collect cells of the right size.

(P2) FSC-A vs. FSC-H plot to collect single cells only.

(P3) CD31/45-FITCH vs. SSC-A plot to exclude endothelial cells and immune cells through negative selection.

(P4) CD56-Brilliant Violet vs. CD34-APC plot to distinguish 34^+ cells (red box) and 34^- cells (blue box).

(P5) CD34-APC vs. FMO CD82-PE-Vio770 plot to distinguish between the $CD82^+$ cells and $CD90^-$ cells of the CD34- cells from (P4). As this is the FMO CD82-PE-Vio770 there are no $CD82^+$ cells (blue box).

Note: MuSC and FAP engage in the cell cycle within 24–48 h and 48–72 h following sorting, respectively. This will lead to exponential growth in cell numbers within 5–10 days. If the cells grow slowly FGF 1:5000 can be added to the growth media. As the FGF-growth media should not be older than one week we recommend making small stocks e.g., by adding 2 µL FGF to 10 mL growth media. Always warm the media or PBS in 37°C water bath before adding it to the cell culture. When changing media make sure to eject to the wall of the well to avoid disturbing the interception of the cells. Store the media at 4°C for up one month when it is not being used.

Plating cells for detection of cell proliferation using EdU

Timing: 24–48 h for MuSCs, 48–72 h for FAPs

For experiments of cell proliferation, we recommend using the Click-iT™ EdU cell proliferation kit for imaging (Thermo Fischer Scientific). For full protocol please see link.

97. Plate the cells at density of approximately $1 \times 10^4/cm^2$.
    a. Add HAMS $F10^+$ wash media at an appropriate amount for the well size choosen.

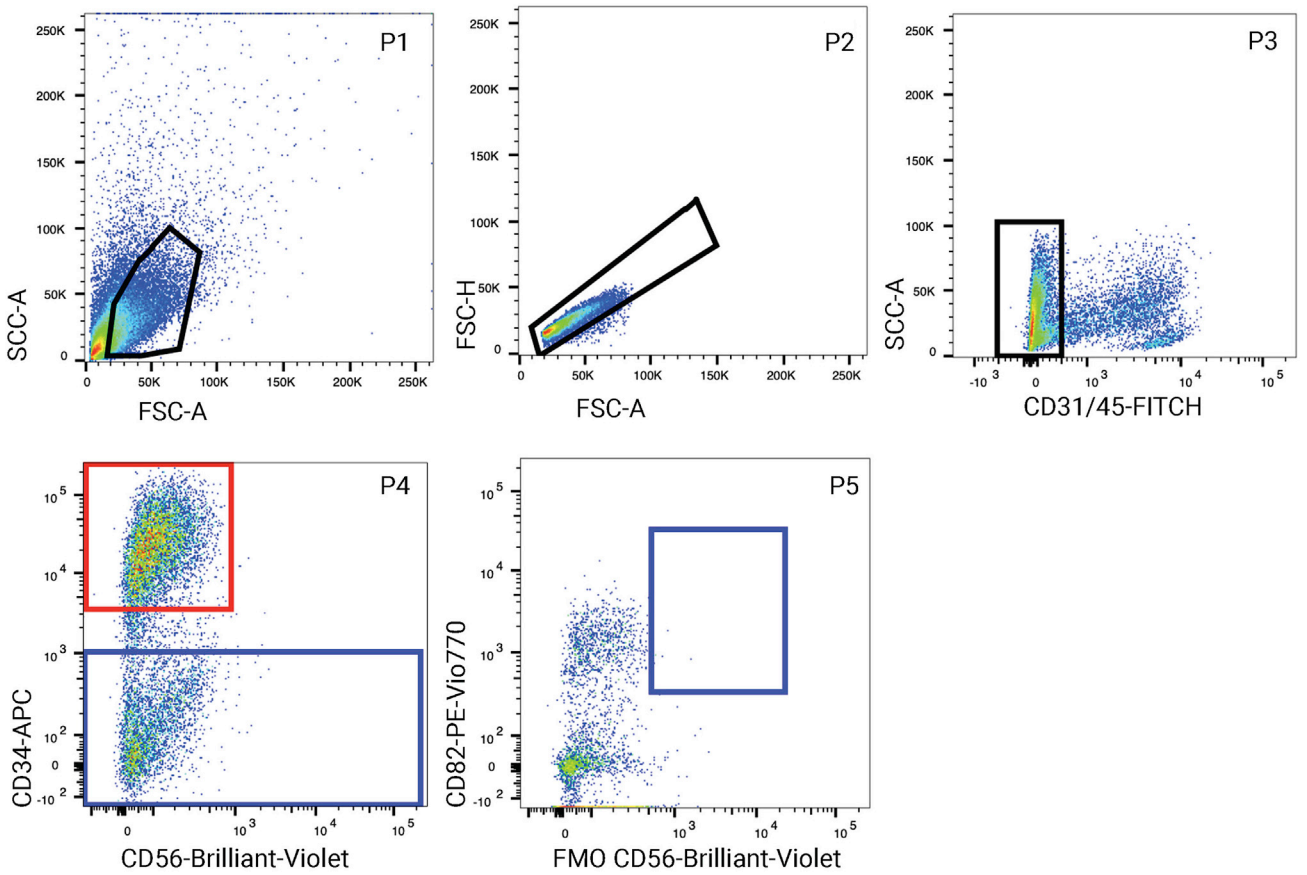

Figure 8. Representative FACS plots for FMO control for CD56-Brilliant-Violet 421

(P1) FSC-A vs SCC-A plot to collect cells of the right size.

(P2) FSC-A vs. FSC-H plot to collect single cells only.

(P3) CD31/45-FITCH vs. SSC-A plot to exclude endothelial cells and immune cells through negative selection.

(P4) CD56-Brilliant Violet vs. CD34-APC plot to distinguish 34^+ cells (red box) and 34^- cells (blue box).

(P5) FMO CD56-Brilliant-Violet vs. CD82-PE-Vio770 plot to distinguish between the $CD56^+$ cells and $CD56^-$ cells of the $CD34^-$ cells from (P4). As this is the FMO CD56-Brilliant-Violet there are no $CD56^+$ cells (blue box).

98. Add EdU from the Click-iT™ EdU cell proliferation kit in HAMS $F10^+$ wash media at concentration of 10 μM upon plating of the cells. Store the media at 4°C for up one month when it is not being used.

Pause Point: 24–48 h for MuSCs, 48–72 h for FAPs.

In our hands it is not necessary to refresh the EdU medium unless cells are grown for longer time periods. However, for prolonged experiments, fresh EdU can be added.

99. Aspirate the media.
100. Fix the cells using 4% paraformaldehyde for 5 min.

⚠ CRITICAL: HAZARD SYMBOL! For details please see manufacturer's SAFETY DATA SHEET on hazard statements and precautionary Statements, prevention using this link.

101. Wash the cells ×3 using 1 × PBS.
102. For detection please follow manufacturer's instruction.

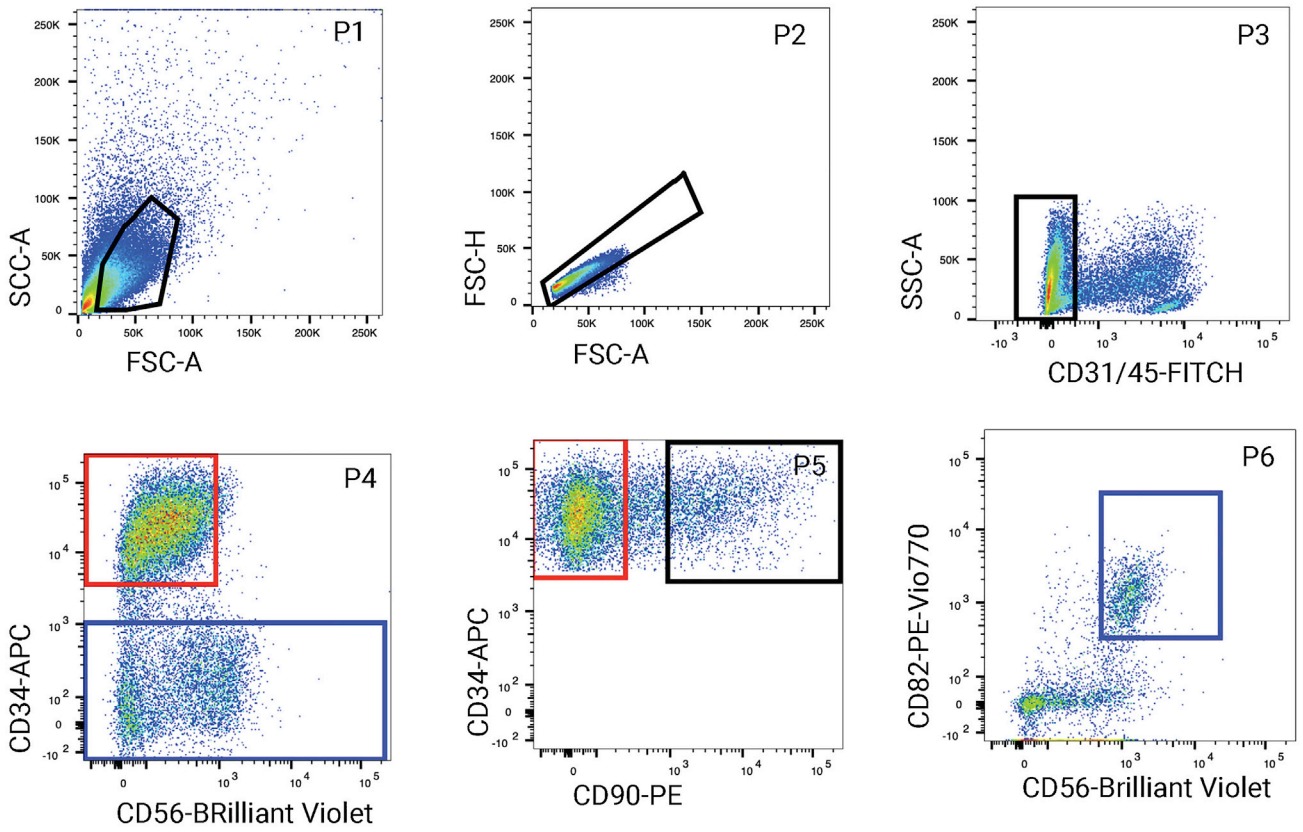

Figure 9. Representative FACS plots for gating strategy for sorting of human MuSCs and FAPs following full staining

(P1) FSC-A vs SCC-A plot to collect cells of the right size.

(P2) FSC-A vs. FSC-H plot to collect single cells only.

(P3) CD31/45-FITCH vs. SSC-A plot to exclude endothelial cells and immune cells through negative selection.

(P4) CD56-Brilliant Violet vs. CD34-APC plot to distinguish 34^+ cells (red box) and 34^- cells (blue box).

(P5) CD90-PE vs. CD34-APC plot to distinguish between the $CD90^+$ cells (red box) and $CD90^-$ cells (black box) of the $CD34^+$ cells from (P4). These are the FAPs.

(P6) CD56-Brilliant Violet vs. CD82-PE-Vio770 plot to collect the $CD56^+CD82^+$ cells from the $CD34^-$ population of P4. These are the MuSCs (blue box).

Note: Fixed cells can be stored in PBS at 4°C until further analysis. If the fixed cells are stored for multiple weeks remember to add PBS to the cells regularly as this will evaporate over time. We have experienced successful staining on fixed cells after one year of storage. However we have not tested the durability of the fixed cells.

Differentiation of MuSCs

Timing: 5–8 days (for step 103)

These steps allows the cells to differentiate to form myotubes.

103. Allow the cells to grow to approximately 95% confluence.
104. Wash the cells once using warm sterile 1×PBS.
105. Add myogenic differentiation media. Add media in a volume appropriate for the well size chosen.
106. Replace the media with fresh media every 2 days. Store the media at 4°C for up one month when it is not being used.
107. Observe the differentiation of the cells on a regular basis using a light microscope.

Table 4. Percentage of each cell population

Population	Percentage of parent population
P1: Cells	35%
P2: Single cells	98%
P3: $CD45^{-}CD31^{-}$	78%
P4: $CD34^{+}$	67% (20%–75%)
P5: $CD34^{-}$	28%
P6: $CD90^{-}$	73% (15%–85%)
P7: $CD90^{+}$	17% (15%–85%)
P8: $CD82^{+}CD56^{+}$	23% (5%–40%)

Percentage of each cell population (eExpected range in parantheses) to fall within the gates. The expected ranges are highly dependent on the proper digestion of the tissue and the donor origin.

Note: Time for full differentiation is expected to be 5–8 days. Upon differentiation the MuSCs will elongate and fuse into multinucleated myotubes.

108. Fix the cells using 4% paraformaldehyde for 5 min once the cells are fully differentiated.

⚠ CRITICAL: HAZARD SYMBOL! For details please see manufacturer's SAFETY DATA SHEET on hazard statements and precautionary Statements, prevention using this link.

109. Wash the cells ×3 using 1×PBS.

Note: Fixed cells can be stored in PBS at 4°C until further analysis. If the fixed cells are stored for multiple weeks remember to add PBS to the cells regularly as this will evaporate over time. We have experienced successful staining on fixed cells after one year of storage. However we have not tested the durability of the fixed cells.

Differentiation of FAPs

Timing: 5–6 days for fibrogenic differentiation, 14 days for adipogenic differentiation (for step 110)

This allows the FAPs to undergo fibrogenic or adipogenic differentiation.

110. Allow the cells to grow to approximately 70% for fibrogenic differentiation and 95% confluence for adipogenic differentiation.
111. Wash the cells once using warm sterile 1×PBS.
112. Add proper differentiation media for either fibrogenic or adipogenic differentiation of FAPs at a volume appropriate for the well size chosen.

Note: For adipogenic differentiation one may use either premade complete adipogenic differentiation media or self-mixed adipogenic media as described under materials and equipment.

113. Replace the media with fresh differentiation media every 2 days. Store the media at 4°C for up one month when it is not being used.

Note: Observe the cells on a regular basis using a light microscope. Time for full differentiation is expected to be 5–6 days for fibrogenic differentiation and 14 days for adipogenic differentiation. Store the media at 4°C when it is not being used.

114. Fix the cells using 4% paraformaldehyde for 5 min.

⚠ CRITICAL: HAZARD SYMBOL! For details please see manufacturer's SAFETY DATA SHEET on hazard statements and precautionary Statements, prevention using this link.

115. Wash the cells ×3 using PBS.

Note: Fixed cells can be stored in PBS at 4°C until further analysis. If the fixed cells are stored for multiple weeks remember to add PBS to the cells regularly as this will evaporate over time. We have experienced successful staining on fixed cells after one year of storage. However we have not tested the durability of the fixed cells.

EXPECTED OUTCOMES

A successful mechanical and enzymatical digestion of muscle tissue followed by a successful incubation of antibodies results in clear cell populations during FACS, which can be distinguished as $CD34^{-}CD56^{+}CD82^{+}CD45^{-}CD31^{-}$ MuSCs and $CD34^{+}CD56^{-}CD45^{-}CD31^{-}$ FAPs (please see gating strategy, Figures 5, 6, 7, 8, and 9). If successful and proper gating strategy has been made using compensation samples and FMO controls, the cell populations fall within these gates with only very little adjustment of the gates needed. Of the mononuclear cell pool analyzed by the sorter the FAPs are generally two to four fold more abundant compared to MuSCs.[5] MuSCs are expected to account for 6%–8% of the cells (20–120 cells/mg).[5,6] The FAPs are expected to account for 25%–50% of the cells (50–300 cells/mg).[5,6] There is linearity between the number of isolated MuSCs and FAPs and the sample size as illustrated by Jensen et al.[5]

The majority of the cells are not actively engaged in the cell cycle before or during the isolation but will naturally be activated by the procedure. Our optimal incubation time with EdU for detection of MuSC and FAP proliferation is 24–48 h and 48–72 h, respectively.[1]

Time for full differentiation is expected to be 5–8 days for myogenic differentiation of MuSC, 5–6 days for fibrogenic differentiation of FAPs and 14 days for adipogenic differentiation of FAPs. *As this may vary it is important to observe the cells using a light microscope on a regular basis.* Following myogenic differentiation of MuSCs analysis of myogenic differentiation can be performed by staining for myosin heavy chain or similar markers of myogenic maturation. Following full differentiation of FAPs detection of adipogenic differentiation can be performed using staining for perilipin 1 (or neutral lipid staining such as Oil-Red-O) whereas fibrogenic differentiation can be detected by staining for collagen 1/3 or alpha smooth muscle actin.

This isolation strategy allows for investigation of cellular *in vivo* traits of these progenitors e.g., in context of metabolic disease or myofiber injury.[1,6] Using this protocol, we have successfully isolated MuSCs and FAPS from humans of various ages, health status and following muscle injuries. We have analyzed biopsies obtained by Bergströms needle as well as biopsies obtained *en bloc* during surgery. The number of cells is roughly comparable between the two techniques as previously showed by our group.[5] We used the same FACS template for all the sorts. Only little adjustment of the gates was needed between the samples. We have plated the cells and confirmed the stem cell nature of the cells. The MuSCs are truly myogenic with potential to form myotubes whereas the FAPs can effectively undergo fibrogenic or adipogenic differentiation.[1]

LIMITATIONS

For successful execution of this protocol a basic knowledge on working using sterile technique in tissue culture hood and FACS is an advantage. If cells are to be used for cell culture experiments basic knowledge of cell culture studies is an advantage as well. Lack of knowledge in these fields might compromise the outcome.

The yield of cells obtained by FACS determines the number of experiments which can be performed. The size of the muscle biopsy is the first limitation for this. If the muscle tissue is sampled using the Bergströms needle the size of the biopsy is expected to be 200–300 mg.[2] Effort must be made to collect as much tissue as possible. This further stresses the need for collecting all possible cells by proper washing of the tubes and collection of the drops hanging underneath the cell strainers. To increase the number of possible experiments using the cells from the sort, we recommend using 96 well half area plates if applicable. Alternatively, to obtain a greater yield of cells one can switch to tissue collected by a surgeon *en bloc* if suitable and possible.

The enzymatic and mechanical digestion is key to high yield.[3] The surface area for the enzymes to work on decreases if the muscle tissue is not minced properly. This limits the number of cells released and may leave undigested muscle tissue. Undigested tissue will be withdrawn in the cell strainers leading to decreased cell yield and challenge the single cell suspension. For optimal isolation procedure with high yield of cells, it is essential to obtain a well digested single cell suspension. Therefore, the muscle tissue must be digested and filtered through cell strainers. Large amounts of connective and adipose tissue as well as undigested muscle fragments may lead to clogging during digestion on the GentleMACS or clogging of the syringe or the cell strainers. Obtaining a well digested and single cell suspension is further important to avoid clogging of the narrow tubes in the FACS machine. Clogging of the FACS machine may lead to the collection tube being filled with PBS which may cause floating and loss of cells. Re-sorting of the sample must be done which increases the risk of dead or dying cells and increases the risk of a lower yield of cells. Effort must be made to remove connective and adipose tissue from the muscle tissue by dissection and secure full suspension of the sample.

When working with human samples there might be small changes to how the cells appear within the gates. This highlights the need to pay attention to the gates and make small adjustments according to the sample being sorted to ensure that the cell populations are always centered in the middle of the gates.

Prolonged storage of the sample at −80°C may decrease the cell viability. However, cell viability following freezing has proven to be 97% for tissue collected by Bergströms needle and 94% for surgically removed muscle tissue.[5] We find that if cells have been stored for a very long time (several months) the cells take longer time to enter the cell cycle or do not enter the cell cycle at all. We therefore recommend sorting the frozen samples within 3–4 weeks.

It is important to always handle samples and cells using sterile technique to avoid infection of cell cultures.

Finally, we have provided markers here for sorting quiescent human MuSCs and FAPs. The extent to which these markers also capture activated, cycling and differentiating cells are yet to be fully described. Thus, it is possible that the utilization of these markers may omit fractions of cycling or differentiating MuSCs or FAPs, which can affect downstream analyses.

TROUBLESHOOTING

Problem 1

Clogging of the C-tube blades may occur from debris of fatty or fibrosus tissue, point 7.

Potential solution

Pause the GentleMACS dissociator. Try to liberate the blades by gently shaking the C-tube. If the blades are still clogged move the C-tube to a ventilated tissue culture hood and gently remove the debris using a pipette before continuing the procedure on the GentleMACS dissociator.

Problem 2

Leftover debris might get caught at the tip of needle (Figure 10), point 11.

Potential solution

Gently remove the debris using a tissue wrap. Does clogging occur while the suspension is in the syringe move the plunger up and down to mobilize the clog. If everything else fails, replace the needle.

Problem 3

The filter may clog from debris, point 13.

Potential solution

Pipette up and down to mobilize the clog. Using a pipette tip any visible debris can be removed. It might be necessary to replace the cell strainer. If a replacement of the cell strainer is needed make sure to transfer *all* liquid in the cell strainer as well as liquid hanging underneath the cell strainer to the new cell strainer in order to maximize cell yield.

Problem 4

Low cell yield. In our experience this is most often related to insufficient digestion of the muscle tissue (point 1–21). This was cause a great loss of cells still attached to fiber fragments and extra-cellular matrix as well as increased amount of debris in the final solution. The latter will result in difficult discrimination between positive and negative cell populations as well as a decreased sorting efficiency.

Potential solution

Several steps can by optimized to prevent this; 1) Consider if the removal of connective tissue and fat from the muscle biopsy has been sufficient (point 2), 2) If tissue originate from surgical waste, ensure that no more than 800–1,000 mg of minced tissue is added to each C-tube (point 3), 3) Ensure that the tissue has been sufficiently minced before adding the enzyme solution (point 2), 4) Ensure that correct amounts of enzyme (U/mL) has been added (enzymatic activity will vary from lot to lot), 5) Ensure that step 11 has been performed to enhance complete dissociation to the tissue and release the cells from fiber fragments and remaining extra-cellular matrix.

Problem 5

Low cell viability. This might occur from lack or insufficient use of serum in the media (Wash buffer). Incorrect handeling of the cell solution in the freezing process can result in increased levels of cell rupture and death. Finally, consider the storage time in the freezer (or alternatively immidiatly sort the cells after digestion if possible.

Figure 10. Left over debris getting caught at the tip of the needle

Potential solution

Make sure that no reagents used in the media have expired and to keep media at 4°C when it is not being used. Make sure to add serum (e.g., 10% horse serum) to the media. While this does partly impair the enzymatic digestion of the tissue, the cell viability is greatly enhanced from this addition. Except for the enzymatic digestion in the gentleMACS, make sure to keep the cell suspension on ice to preserve cell function.

Make sure to always add StemMACS cryopreservation buffer (or similar freezing buffer) to the cells and use an appropriate freezing container (point 18–20). The thawing of the cells can lead to low cell viability. Make sure to thaw cells quickly in a water bath and transfer the cells to HAMS $F10^{+}$ wash media immediately (point 22). Consider if all tubes and filters have been washed sufficienty to collect *all cells possible* throughtout the protocol.

In our experience the cells should be used for FACS within a month for best viability and ability of the cells to enter the cell cycle. Make sure to sort the cells directly into media containing serum if possible and plate the cells as soon as possible after sorting.

Problem 6

Contamination of isolated cultured cells.

Potential solution

Consider if sterile procedures have been followed at all times throughout the protocol. To help avoid contamination Penicillin-streptomycin (penstrep) should be added to all media being used. Consider if the cell sorter has been properly cleaned and aseptic procedures has been performed before usage. In general, primary cells should be cultivated separate from cell-lines since the former is difficult to maintain completely sterile.

Problem 7

Impure populations in culture. This issue often arises from either poor tissue digestion or improper cell sorter settings.

Potential solution

In relation to improper tissue digestion, please consult the points in Problem 4, as the same considerations are important here. If the tissue is not sufficiently digested and single cells liberated this will result in large amounts of debris in the sort, which increases the likelihood of impure sorted cell populations. As for the sorter, ensure that the settings on the sorter are enforcing a high purity (at the expense of high yield) of the sorted cell populations (point 74–89).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jean Farup, jean@biomed.au.dk.

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate any databases. Detailed sorting strategy is available from corresponding author on request.

ACKNOWLEDGMENTS

We would like to thank the FACS core facility at Aarhus University for assistance in setting up the sorting protocol. This work was supported by the A.P. Møller Foundation, Riisfort Foundation, Toyota

Foundation, and The Independent Research Fund Denmark (DFF – 5053-00195) to J.F. and the Steno Diabetes Center Aarhus, which is partially funded by an unrestricted donation from the Novo Nordisk Foundation. Illustrations are made with a license from biorender.com.

AUTHOR CONTRIBUTIONS

Investigation, T.B.B., J.B.J., and J.F.; Writing – original draft, T.B.B. and J.F.; Writing – review and editing, J.B.J. and N.J.; Funding, J.F. and N.J.; Supervision, J.F. and N.J.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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