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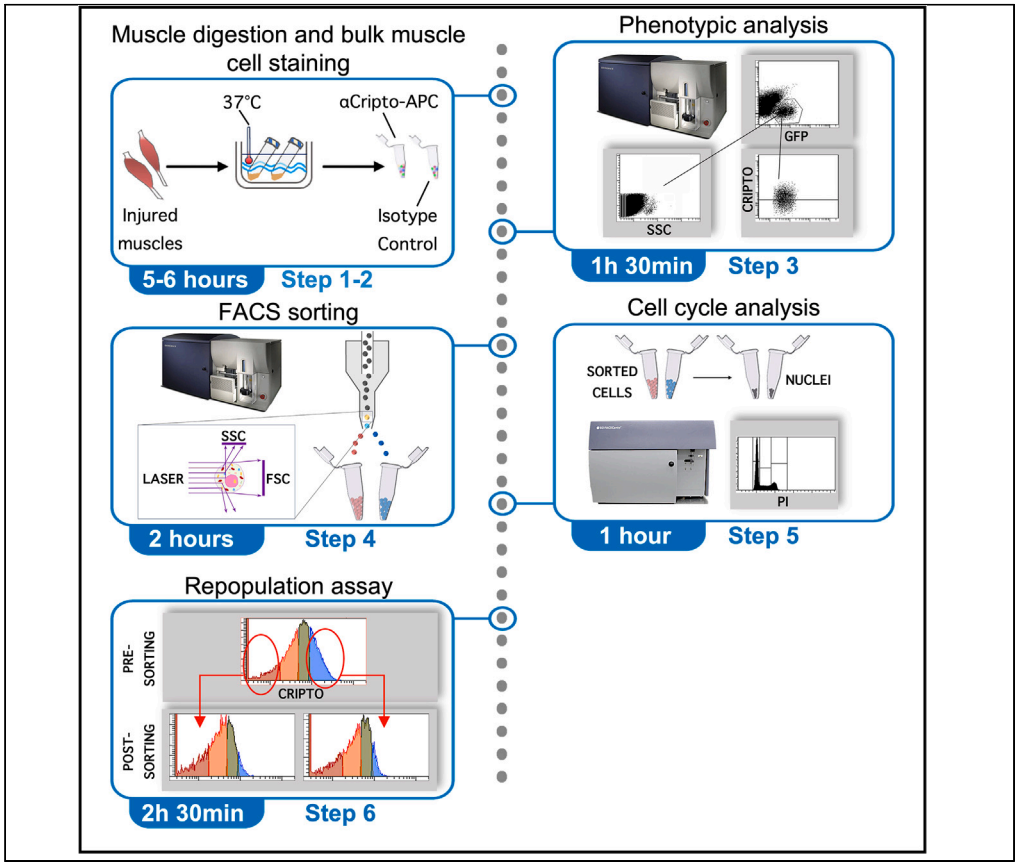
Protocol for characterizing non-genetic heterogeneity and expression dynamics of surface proteins in mouse muscle stem cells using flow cytometry



Here, we present a protocol for investigating the non-genetic heterogeneity of membrane proteins expression within murine muscle stem cell (MuSC) population isolated from injured skeletal muscles. We describe a protocol that employs flow cytometry technology to detect variations in membrane CRIPTO protein levels and ensure measurements standardization. We detail steps for muscle digestion, bulk muscle cell staining, and phenotypic analysis. This approach allows for the identification of MuSC fractions with distinct phenotypic and functional properties.

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

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Highlights

Preparation of bulk muscle cell suspension from injured skeletal muscle tissue

Use of flow cytometry for immunofluorescence and phenotypic analysis of MuSCs

Sorting MuSCs by FACS based on CRIPTO protein levels to identify cell fractions

Analysis of MuSC cell cycle and reversible states through *ex vivo* repopulation assays

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Protocol for characterizing non-genetic heterogeneity and expression dynamics of surface proteins in mouse muscle stem cells using flow cytometry

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SUMMARY

Here, we present a protocol for investigating the non-genetic heterogeneity of membrane proteins expression within murine muscle stem cell (MuSC) population isolated from injured skeletal muscles. We describe a protocol that employs flow cytometry technology to detect variations in membrane CRIPTO protein levels and ensure measurements standardization. We detail steps for muscle digestion, bulk muscle cell staining, and phenotypic analysis. This approach allows for the identification of MuSC fractions with distinct phenotypic and functional properties.

For complete details on the use and execution of this protocol, please refer to Guardiola et al.[1]

BEFORE YOU BEGIN

Our protocol describes the analysis of the Muscle Stem Cells (MuSCs) population isolated from injured skeletal muscles of Pax7nGFP reporter mice,[2] with a specific focus on the heterogeneity of the cell surface CRIPTO protein levels in activated MuSCs.[1,3] Pax7nGFP reporter mice enable MuSC population identification, isolation, and characterization *in vivo* using fluorescence-activated cell sorting (FACS) based on the expression of green fluorescent protein (GFP) driven by the Pax7 promoter, as Pax7 is a marker of both quiescent and activated MuSCs. Alternatively, MuSC population can be isolated from wildtype mice using sets of specific antibodies.[4] The expression levels of surface CRIPTO protein in the GFP+ population are analyzed through the use of conjugated anti-CRIPTO antibodies. This approach enables detecting and tracing the dynamic heterogeneity of activated MuSCs, as well as the physical isolation of specific MuSCs fractions or subpopulations based on the expression levels of cell surface membrane proteins such as CRIPTO. While our protocol primarily focuses on analyzing CRIPTO expression levels, it can potentially be adapted to different cell surface membrane proteins using conjugated antibodies. This versatility enables the monitoring of sorted cells to assess their behavior and potential transition between different functional states. Moreover, we highlight the significance of sorting strategies in distinguishing between stable, irreversible and dynamically reversible cell states within the MuSC population. In this protocol, we utilize hindlimb skeletal muscles previously injured by cardiotoxin (CTX) intramuscular injection for the isolation and study of MuSCs.[5] The injection of CTX allows for the reproducible induction of cellular and molecular mechanisms involved in the regeneration process, stimulating MuSCs to exit their quiescent state. In this context, CRIPTO is transiently expressed in activated MuSCs, with a peak at day 3 post-injury, while it is undetectable in quiescent (non-injured) muscles.

Institutional permissions

All experiments conducted adhered strictly to institutional guidelines for animal research and received approval from the Department of Public Health, Animal Health, Nutrition and Food Safety of the Italian Ministry of Health. Authorization numbers DM n. 868/2015-PR and 720/2020-PR were obtained in compliance with animal experimentation laws. Researchers intending to utilize this protocol for their own investigations must obtain authorization from their institutions. Additionally, they must adhere to ethical and legal guidelines as stipulated by their local, state, and federal regulations.

Preparation for muscle cells isolation

Timing: 30–60 min

1. Ensure this procedure is conducted under sterile conditions and using pre-sterilized materials.
2. Essential equipment such as a thermostatic stirring water bath at 37°C, a vacuum system, pipettes and an electronic pipettor must be readily available.
3. Harvest hindlimb skeletal muscles previously injured by cardiotoxin (CTX) intramuscular injection[5] for subsequent mechanical and enzymatic dissociation.
4. Prepare the necessary solutions as outlined in the 'materials and equipment' section before proceeding with each step of the protocol.
5. Prepare the Digestion Mix as follow:
   a. Weigh Dispase II and Collagenase A into a 50 mL tube.
   b. Add PBS 1×.
   c. Vortex to dissolve the lyophilized enzymes.
   d. Add $CaCl_2$, $MgCl_2$, and DNaseI.
   e. Adjust the volume with PBS 1×.
6. Prepare the Cell Resuspension Solution as follow:
   a. Prepare a 20% BSA solution by weighing 1 g of BSA in 20 mL of PBS 1×.
   b. Vortex to dissolve the BSA.
   c. Add the solution to 500 mL HBSS.
   d. Add the penicillin/streptomycin (P/S) 1×.
7. Prepare the Staining, FACS and Cell Collection solutions.
8. Sterilize all the solutions using 0.22 μm filters before use.
9. Store the solutions on ice or at 4°C until use.

Preparation for fluorescence-activated cell sorting (FACS)

BD FACSAria III setup

Timing: 45 min

10. Turn on instrument and perform fluidic startup to remove bubbles and ensure the stability of the stream.
11. Install the 80 μm nozzle and verify that it matches with the Cytometer Configuration.
12. Apply the cleaning and sterilization procedure: 10 min with de-ionized water (DI water), 5 min of 70% ethanol, 10 min with DI water, sterile sheath fluid or PBS until the acquisition.
13. Stabilize the frequency and amplitude in the Break-off window and ensure the stability of the sheath stream.
14. Before loading and acquiring all samples, the BD FACSAria III cell sorter undergoes a routine calibration using Sphero Rainbow Calibration Particles to ensure the linearity of laser emission and fluorescence detection for comparison between different experiments.
15. Before each cell sorting, the drop delay of the instrument is calibrated with FACS Accudrop Beads to guarantee the accuracy of cell recovery with high purity percentages.

BD FACSCanto II setup

Timing: 30 min

16. Turn on instrument and perform fluidic startup to remove bubbles.
17. Apply the cleaning procedure: 10 min with DI water at a medium flow rate to ensure the absence of residues or contaminants.
18. Before loading and acquiring all samples, the BD FACSCanto II analyzer, similar to the BD FACSAria III, undergoes routine calibration using Sphero Rainbow Calibration Particles to ensure the linearity of laser emission and fluorescence detection for comparison between different experiments.

Note: The setup procedures described for both instruments have been refined based on the manufacturer's guidelines and tailored to experimental needs. Similar procedures can be adapted for instruments from different brands, following the respective manufacturer's instructions. The use of calibration beads is recommended to ensure the accuracy and reproducibility of results.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse Cripto allophycocyanin MAb	Bio-Techne - R&D Systems	Cat# FAB1538A
Rat IgG2B APC-conjugated isotype control	Bio-Techne - R&D Systems	Cat# IC013A
Chemicals, peptides, and recombinant proteins		
Dispase II	Roche	Cat# 10887800
Collagenase A	Roche	Cat# 10103586001
DNase I	Roche	Cat# 11284932001
Hank's balanced salt solution (HBSS)	Gibco	Cat# 14170-088
Bovine serum albumin (BSA)	Sigma-Aldrich	Cat# A4503
Penicillin-streptomycin (P/S)	Gibco	Cat# 15140-122
Fetal bovine serum (FBS)	Euroclone	Cat# ECS5000L
Goat serum	Merck	Cat# G9023
Propidium iodide	Sigma-Aldrich	Cat# P4864
PureLink RNase A	Invitrogen	Cat# 12091021
NaCl	Sigma-Aldrich	Cat# S5886
KCl	Sigma-Aldrich	Cat# P5405
Na_2HPO_4	Sigma-Aldrich	Cat# S5136
KH_2PO_4	Sigma-Aldrich	Cat# P5655
EDTA, disodium salt	Baker	Cat# 1073
$CaCl_2$	Sigma-Aldrich	Cat# C5670
$MgCl_2$	Sigma-Aldrich	Cat# M2393
PBS	Invitrogen	Cat# 14190-094
Sodium citrate dihydrate	Sigma-Aldrich	Cat# C8532
Citric acid	Sigma-Aldrich	Cat# C2404
Nonidet P-40	Thermo Fisher Scientific	Cat# J19628.AP
Experimental models: Organisms/strains		
Mouse: Tg:Pax7-nGFP – 6–8 weeks old males	The Jackson Laboratory	MGI:5308730
Software and algorithms		
BD FACSDiva software (v8.0.1)	BD Biosciences	https://www.bdbiosciences.com/en-us/products/software/instrument-software/bd-facsdiva-software
Other		
Syringe filter, 25 mm, AcrylicHousing, 0.2 μm CA membrane, sterile	GVS	Cat# FJ25ASCCA002DL01
Swann-Morton no 18 sterile carbon steel blades	HandyProducts	Cat# 0223

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Pipette filter tips, size 1,000 µL	Merck	Cat# AXYTF1000RS
Cell strainers, pore size 100 µm	Corning	Cat# CLS431752
Cell strainers, pore size 70 µm	Corning	Cat# CLS431751
Cell strainers, pore size 40 µm	Corning	Cat# CLS431750
15 mL centrifuge tubes	Corning	Cat# CLS430791-500EA
50 mL centrifuge tubes	Corning	Cat# CLS430829-500EA
Carbon steel surgical blades sterile 18	Swann-Morton	Cat# 0223
Fine scissors – curved/sharp-sharp/10.5 cm	2Biological Instruments	Cat# 14061-10
Pierce microcentrifuge tubes, 1.5 mL	Thermo Scientific	Cat# 69715
FACS tubes	Falcon	Cat# 352054
BD Plastipak 1 mL syringe with detached BD Microlance 3 needle	BD Biosciences	Cat# 303176
Syringe Filcons 70 µm	BD Biosciences	Cat# 340606
FACS Accudrop beads	BD Biosciences	Cat# 345249
Sphero Rainbow calibration particles	BD Biosciences	Cat# 559123
Laboratory water bath	Euroclone	Cat# SWB, SRB
BD FACSAria III	BD Biosciences	https://www.bdbiosciences.com/en-at/products/instruments/flow-cytometers/research-cell-sorters/bd-facsaria-iii
BD FACSCanto II	BD Biosciences	https://www.bdbiosciences.com/en-de/products/instruments/flow-cytometers/clinical-cell-analyzers/facscanto

MATERIALS AND EQUIPMENT

PBS 1×

Reagent	Final concentration	Amount
NaCl	137 mM	8 g
KCl	2.7 mM	0.2 g
Na_2HPO_4	10 mM	1.44 g
KH_2PO_4	1.8 mM	0.245 g
Distilled H_2O	N/A	N/A
Total	N/A	1 L

Store at 25°C after sterilized for up to 6 months.

EDTA

Reagent	Final concentration	Amount
EDTA	0.5 M	93,06 g
Distilled H_2O	N/A	N/A
Total		500 mL

Adjust to pH 8.0 with NaOH. Store at 25°C after sterilized for up to 6 months.

Digestion Mix for 1 sample (6 hindlimb muscles)

Reagent	Final concentration	Amount
Dispase II (1 U/mg)	3 U/mL	N/A
Collagenase A	2 U/mL	N/A
CaCl2 (50 mM)	0.4 mM	32 µL
MgCl2 (1 M)	5.0 mM	20 µL
DNaseI (10 mg/mL)	10 ng/µL	4 µL
PBS 1×	N/A	N/A
Total	N/A	4 mL

Prepare the solution immediately before use, avoiding freezing.

Cell Resuspension Solution		
Reagent	Final concentration	Amount
Pen/Strep	1×	5 mL
BSA	0.2%	1 g
HBSS	N/A	N/A
Total	N/A	500 mL
Store at 4°C, up to 3 months.		

Staining Solution		
Reagent	Final concentration	Amount
Goat serum or FBS	1%	100 µL
PBS 1×	N/A	9.9 mL
Total	N/A	10 mL
Aliquot and store at −20°C, up to 3 months.		

FACS Solution		
Reagent	Final concentration	Amount
Goat serum or FBS	10%	1,5 mL
DNaseI (10 mg/mL)	100 ng/µL	150 µL
EDTA	2 mM	60 µL
PBS 1×	N/A	N/A
Total	N/A	15 mL
Aliquot and store at −20°C, up to 3 months.		

Cell Collection Solution		
Reagent	Final concentration	Amount
FBS	20%	3 g
PBS 1×	N/A	N/A
Total	N/A	15 mL
Aliquot and store at −20°C, up to 3 months.		

Na-Citrate buffer (0.1 M, pH 6)		
Reagent	Final concentration	Amount
Sodium Citrate dihydrate	0.0825 M	24.269 g
Citric Acid	0.0175 M	3.358 g
Distilled H_2O	N/A	N/A
Total	N/A	1 L
Adjust solution to pH 6 using 0.1 N HCl. Store the solution at 4°C, up to 6 months.		

Cell Cycle Solution		
Reagent	Final concentration	Amount
Na-Citrate buffer (10%)	0.1%	10 µL
Nonidet P-40 (5%)	0.05%	10 µL
Propidium Iodide (2 mg/mL)	50 µg/mL	25 µL
RNase (1 mg/mL)	0.2 µg/µL	100 µL
PBS 1×	N/A	855 µL
Total	N/A	1 mL
Prepare the solution immediately before use.		

STEP-BY-STEP METHOD DETAILS

Bulk muscle cell suspension preparation

Timing: 4 h 30 min

This step generates a bulk cell suspension containing a heterogeneous mixture of cells, including MuSCs.

1. Muscle dissociation:
   a. Use a scalpel and scissors with curved tips to finely mince the harvested injured muscles until no whole pieces are visible.
   b. Transfer the minced muscles from the mouse (6 hindlimb muscles) into a 50 mL tube containing 4 mL of Digestion Mix. Keep the tubes at 25°C–26°C.

⚠ CRITICAL: Avoid mixing muscles from different mice within the same tubes, ensuring separate processing for each specimen.

2. Incubation.
   a. Incubate for 90 min at 37°C under agitation in a thermostatic stirring water bath (140 rpm).
   b. Pipette the mixture halfway through the incubation period (after 45 min) with a P1000 pipette tip with filter.

⚠ CRITICAL: In point 2a, place the tubes at an inclined position for incubation, facilitating cell shacking and preventing cells from settling at the bottom. Do not incubate minced tissue in digestion mix for longer than 90 min.

3. Filtration.
   a. Add Cell Resuspension Solution to reach a maximum volume of 30 mL and resuspend the cells.
   b. Filter the contents of the tube through 100 μm cell strainers.
   c. Centrifuge at 400 × *g* for 6 min at 25°C–26°C.
   d. Aspirate the supernatant, leaving a small residual volume and taking care not to disturb the pellet.
   e. Resuspend the cells in Cell Resuspension Solution up to a maximum volume of 30 mL.
   f. Filter the contents of the tube through 70 μm cell strainers.
   g. Centrifuge at 400 × *g* for 6 min at 25°C–26°C.
   h. Aspirate the supernatant, leaving a small residual volume.
   i. Add Cell Resuspension Solution to reach a maximum volume of 30 mL and resuspend the cells.
   j. Filter the contents of the tube through 40 μm cell strainers.
   k. Centrifuge at 400 × *g* for 6 min at 25°C–26°C.
   l. Aspirate the supernatant and adjust the volume between 1 and 1.5 mL using Cell Resuspension Solution to proceed to the next step.

Note: Maintaining a consistent temperature is crucial to prevent fluctuations that may affect protein trafficking. It is advisable to conduct all steps at 25°C–26°C to avoid thermal shocks and ensure optimal conditions for the procedure.

Immunofluorescence for FACS analysis

Timing: 1–1 h 30 min

This step outlines the procedure for sample preparation and staining with specific antibodies, which precedes sample analysis by the flow cytometer

4. Dilute the appropriate volume of Isotype Control and CRIPTO-APC antibody (1:10, v/v) in Staining Solution and store it on ice in the dark.
5. Transfer the cell suspension into two 1.5 mL tubes: one designated for the Isotype Control and the other for the conjugated antibody. See Note below.
6. Centrifuge cell suspensions at 400 × *g* for 4 min at 25°C–26°C.
7. Aspirate the supernatant.

Note: At point 5, consider transferring a smaller volume (e.g., one-tenth) of the resuspended cells for the Isotype Control to potentially improve the final yield of sorted cells.

8. Resuspend the cell pellets with the diluted antibody or isotype solution prepared in point 4.
9. Incubate the resuspended cells on a rotating wheel at 25°C–26°C in the dark for 45 min.
10. Add FACS Solution (at least 500 μL per sample) and filter with Syringe Filcons into FACS loading tubes.
11. Store the tubes in the dark on ice or at 4°C until the Flow Cytometry acquisition of samples to prevent degradation and loss of fluorescence signals from occurring.

⚠ CRITICAL: Maintain a consistent temperature, as fluctuations can affect membrane protein trafficking and expression levels.

Phenotypic analysis by flow cytometry

Timing: 1 h 30 min

This step involves flow cytometry analysis performed using the BD FACSAria III to identify GFP-positive MuSCs and assess surface CRIPTO protein expression levels. Additionally, it shows a correlation analysis between SSC-A, a key morphological parameter, and CRIPTO protein levels.

12. Set up the worksheet on BD FACSDiva software and define the appropriate dot plots, as follows:
    a. Dot plot of FSC-A vs. SSC-A parameters detection: defines the physical parameters (size and complexity) of the heterogeneous sample (Figure 1A).
    b. Dot plot of FSC-H vs. FSC-A parameters detection: identify single cell and exclude aggregates (Figure 1B).
    c. Dot plots fluorophore such as PerCP-Cy5.5 vs. GFP fluorescence, SSC-A parameter vs. GFP fluorescence, and FSC vs. GFP fluorescence: identify the MuSCs- GFP positive cell population (Figure 1C, upper panel).
    d. Dot plot CRIPTO APC fluorescence vs. GFP fluorescence: enables establishment of the negativity threshold using the control isotype, and allows for the measurement of CRIPTO expression levels on MuSCs- GFP positive cells (Figures 1D and 1E).
13. Load the cells resuspended in FACS solution onto Flow Cytometer and proceed with data acquisition, applying the following gating strategy:
    a. Draw a gate around the heterogeneous live population (P1 population in Figure 1A) on the FSC-A and SSC-A plot.
    b. Isolate single cells and exclude aggregates using the FSC-H and FSC-A dot plot (P2 population in Figure 1B).
    c. Define MuSCs-GFP positive population by plotting PerCP-Cy5.5 vs. GFP fluorescence (P3 population in Figure 1B).
    d. Use the Isotype Control to set the voltages defining the negative threshold of fluorescence and identify the total CRIPTO positive population (P6 population in Figure 1C).
    e. Plot the fraction of GFP-positive cells against APC fluorescence (anti-CRIPTO-APC antibody) to identify the double-positive MuSCs population and determine the gradient of CRIPTO expression level (Figures 1D and 1E).

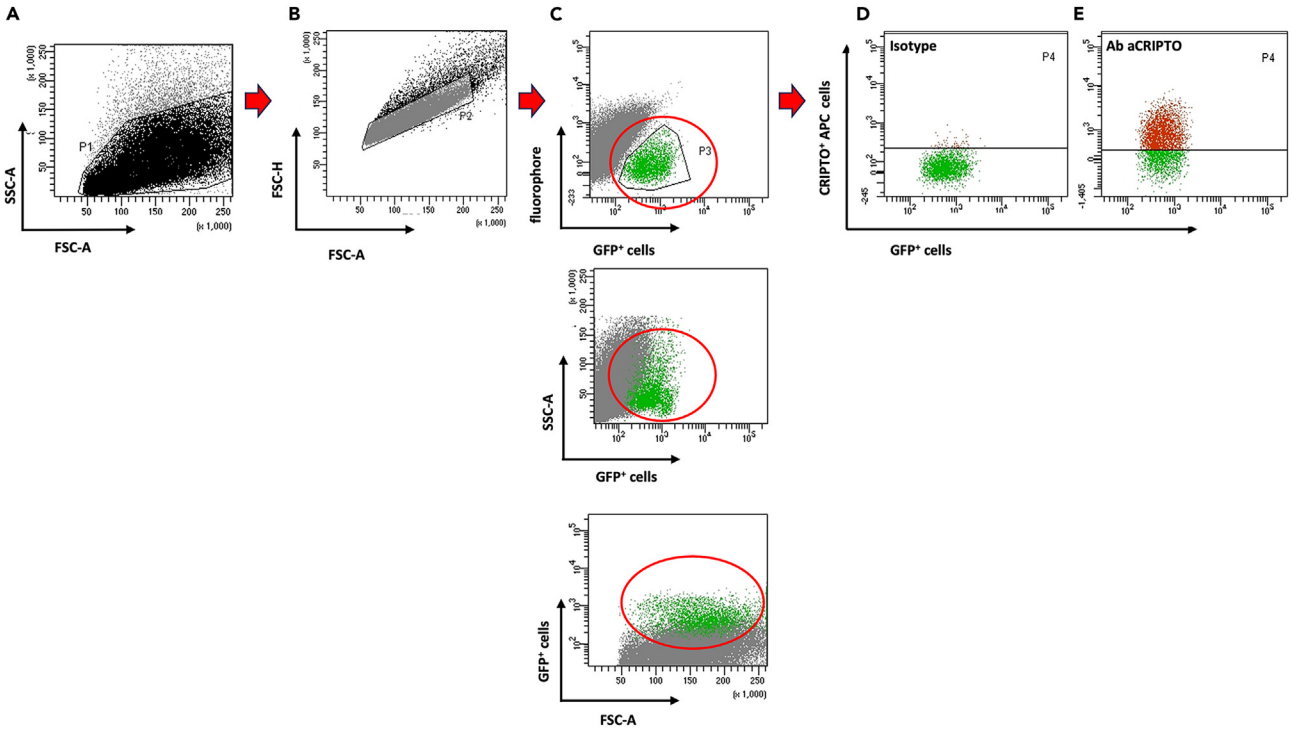

Figure 1. Gating strategy for MuSCs- GFP and CRIPTO expression

(A) SSC-A/FSC-A plot shows the characterized cells population. The P1 gate includes the live population and excludes cell debris.

(B) FSC-H/FSC-A dot plot excludes cell aggregates and select single cells only.

(C) Dot plots used to identify the GFP-positive population in the heterogeneous muscle cells suspension. P2 gate represents GFP cells used to detect CRIPTO expression. Red circles identify GFP positive population.

(D) Isotype control panel to define the negative threshold of fluorescence.

(E) Dot plot showing the heterogeneous distribution of CRIPTO expression on GFP positive MuSCs population. The P6 gate includes double positive GFP/CRIPTO MuSCs population.

Note: considering the heterogeneous nature of muscle cell suspension, PerCP-Cy5.5 vs GFP fluorescence and GFP fluorescence vs SSC-A dot plots provide better resolution of GFP-positive population and improve separation from the negative cells (Figure 1B, middle and upper panel, respectively), compared to the GFP fluorescence vs FSC-A dot plot (Figure 1C, bottom panel). Adopting this gating strategy is recommended, especially in case of low represented populations from *ex vivo* samples.

14. Set up a dot plot of CRIPTO (anti-CRIPTO-APC antibody) vs. GFP.
15. Divide the cell population into four groups, each representing 25% of the total population, based on the intensity of CRIPTO expression.

Note: These will include CRIPTO Positive cells (P4), CRIPTO Negative cells (P3), and two additional fractions with intermediate levels of expression (P5 and P6) (Figure 2A).

16. Obtain the SSC-A mean values and the CRIPTO_APC mean fluorescence intensity values from statistical information automatically provided by the BD FACSDiva software for each of the four fractions.
17. Perform linear correlation analysis between the SSC-A mean values and the mean fluorescence intensity for each fraction (Figure 2B).

Note: SSC provides insights into internal cell complexity, such as nucleus /cytoplasm ratio, cytoplasmic granularity and density, offering direct indications of cell activation state and

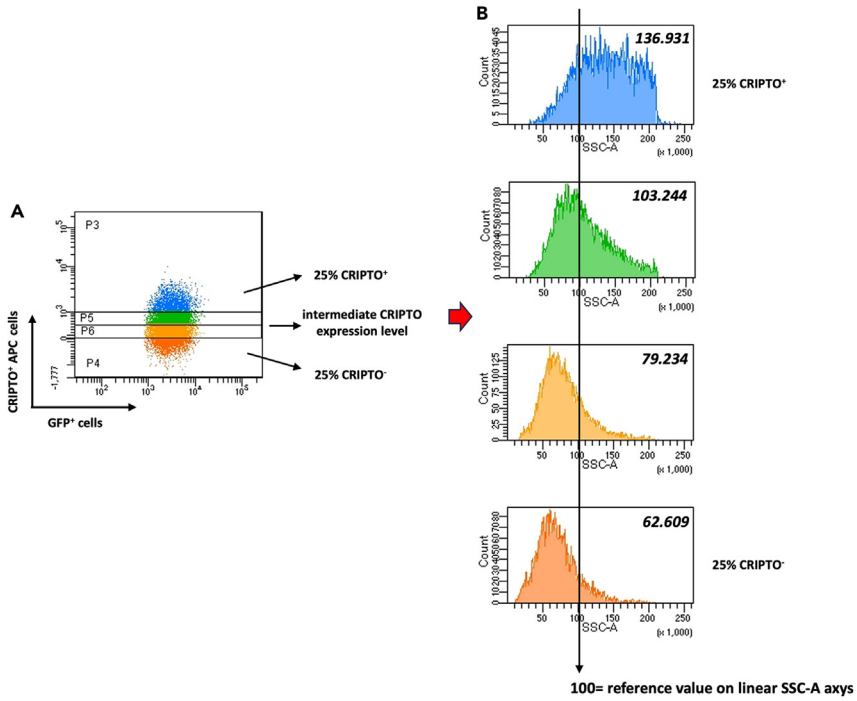

Figure 2. Correlation Between CRIPTO Expression and Morphological Characteristics in MuSCs

(A) Dot plot of CRIPTO (anti-CRIPTO-APC antibody) vs. GFP fluorescence intensity in MuSCs, showing the population divided into four fractions (25% each) based on CRIPTO expression levels.

(B) Histograms of SSC-A (side scatter), each representing one of the fractions obtained from the dot plot analysis. SSC-A mean value is reported for each histogram.

productivity. To observe variations in SSC-A distribution, define a reference value against which to monitor the changes. We have arbitrarily marked this value as 100 along the linear SSC-A scale.

⚠ CRITICAL: Recording a significant number of cells necessary to visualize a homogeneous distribution (at least 50,000 events of P2 population after FSC-A/SSC-A detection and single cells discrimination by FSC-H vs FCS-A, Figure 1B). A low cell count reduces the resolution of SSC-A histograms.

Fluorescence-activated cell sorting (FACS)

Timing: 2 h

This step describes the utilization of flow cytometry for identifying and sorting cell fractions that express varying levels of surface CRIPTO protein. Additionally, it outlines procedures for sorting quality control.

18. Recover CRIPTO Positive and CRIPTO Negative cell fractions by cell sorting.
    a. Classify the cell fractions as CRIPTO positive and CRIPTO negative, representing the 25% of the GFP-positive cell population with the highest fluorescence intensity of CRIPTO-APC and the 25% with the lowest fluorescence intensity, respectively (P15 and P16 populations in Figures 3A and 3B).
    b. Acquire data for all the samples, ensuring that at least 50,000 events from selected populations are recorded.

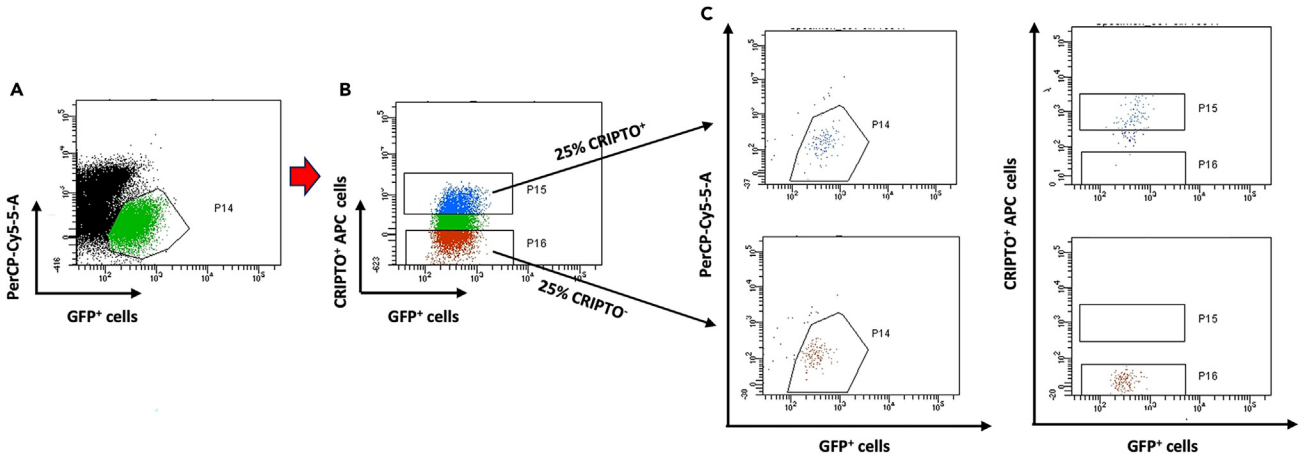

Figure 3. Gating strategy to control sorting purity and quality

(A) Dot plot used to identify GFP-positive population in heterogeneous muscle cells suspension (P14 population).

(B) PE-Cy5.5 vs. GFP fluorescence dot plot used to identify CRIPTO Positive (P15) and CRIPTO Negative (P16) cell fractions.

(C) Control dot plots show sorting result, verifying GFP positivity and correct distribution based on CRIPTO fluorescence intensity for each processed sample.

c. Install the collection tubes (1,5 mL tube) with 300 μL of Cell Collection solution into the collection chamber to collect the selected and sorted cells.
d. In the 'sort menu' select the sort location according to the tubes positions and proceed whit the sorting.
e. Recover a maximum 2500000 cells for each collection tube to avoid overheating or an increase in the percentage of dead cells.
f. While the sorting process is ongoing, keep the already collected cells on ice.

19. For each sample proceed to sorting quality control:
    a. Take a portion of the recovered cells (20/50 μL in PBS).
    b. Verify if the cells are consistently GFP positive.
    c. Verify if the cells are correctly distributed in CRIPTO positive and CRIPTO negative gates before proceeding experimentally (Figure 2C).

⚠ CRITICAL: It is necessary that the emission spectrum of the selected fluorophore is different to that of GFP to avoid applying compensation. For cell acquisition and cell sorting, it is important to stabilize the flow rate at 1.0/1.2 to regulate the acquisition of 3000–5000 events/seconds (evt/sec). Maintaining a regular flow rate is essential, as a high flow rate is associated to high pressure on cells, leading to damage, reduced sorting efficiency, and purity. During sorting quality control, the CRIPTO positive cell fraction may exhibit lower fluorescence intensity compared to the initial sample, possibly due to a natural loss of brightness of the fluorophore and/or the protein turnover. A purity above 85% is considered a good post-sorting recovery.

In accordance with the fluorophores used for antigen detection, cell viability of sorted cells can be assessed using reagents such as 7-aminoactinomycin D (7-AAD, Thermo Fisher Scientific cat. no. A1310) or SYTOX dye (Thermo Fisher Scientific). The choice of staining reagent should align with the fluorophores used for detecting antigens of interest in the cell population being analyzed.

The cell pellet obtained after sorting and centrifugation may be almost invisible. Be careful not to touch the bottom of the tube while removing the supernatant.

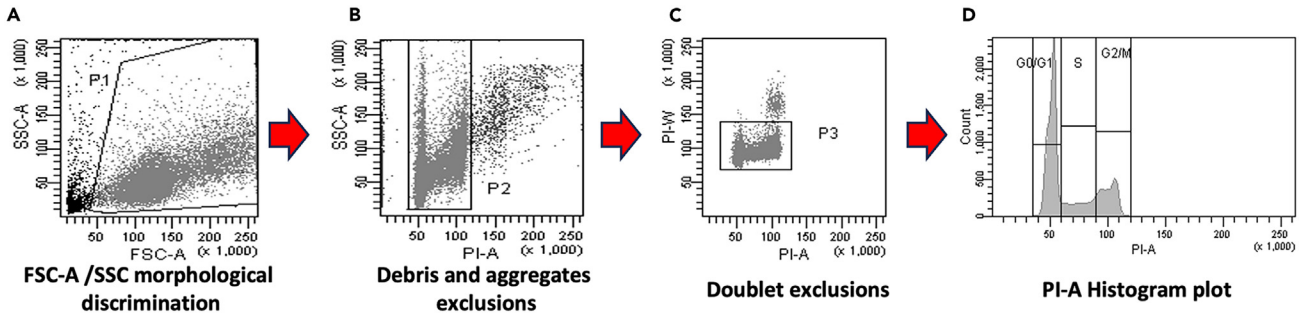

Figure 4. Gating strategy to cell cycle analysis

(A) SSC-A/FSC-A plot shows nuclei population. The P1 gate represents the selected subset for cell cycle analysis.

(B) SSC-A vs. PI-A dot plot enables exclusion of debris, aggregates and the excess of non-specific PI signals, defining P2 population.

(C) PI-W vs. PI-A dot plot enables exclusion of doublets and identification of single cells, defining P3 population.

(D) PI-A linear histogram of the P3 population shows the distribution of single cells across the different cell cycle phases.

Cell cycle analysis by flow cytometry

Timing: 1 h

This step outlines a method for preparing post-sorting cells for cell cycle analysis by directly analyzing the cell nuclei. This cell cycle analysis protocol offers significant advantages for quickly analyzing cell subpopulations poorly represented or subjected to sorting stress, which would otherwise be lost by using the classical ethanol-based procedure. It ensures rapid results by lysing the plasma membrane, reducing the autofluorescence or non-specific cytoplasmic staining.

20. Incubate sorted cell in Cell Collection solution at 25°C–26°C for 15 min to balance the altered membrane charges caused by sorting.
21. Set up the worksheet on BD FACSDiva software and define the correct dot plot for the data visualization and analysis as described below:
    a. Dot plot of FSC-A vs. SSC-A parameters detection: defines the physical parameters (size and complexity) of the sample (Figure 4A).
    b. Dot plots SSC-A vs. PI-A: excludes debris, aggregates and the excess of non-specific propidium iodide (PI) signals (Figure 4B).
    c. Dot plot PI-W vs. PI-A: excludes doublets and identify single cells (Figure 4C).
    d. Histogram plot PI-A: shows the distribution of single cells in different cell cycle phases (Figure 4D).
22. Centrifuge the cells 4 min at 400 × *g*.
23. Aspirate the supernatant.
24. Resuspend cell pellets in 500 µL of Cell Cycle solution and incubate at 25°C–26°C for 15 min.
25. Load the cells onto the Flow Cytometer and proceed with data acquisition, applying the following gating strategy:
    a. Define a gate around the cell population in the FSC-A and SSC-A plot (P1 population in Figure 4A).
    b. Adjust PI fluorescence in linear amplification to resolve the fluorescence peaks of the G0/G1 and G2/M cells individually. Logarithmic amplification may cause the peaks to appear overlapped and compressed.
    c. Show P1 population in SSC-A/PI-A dot plot, setting the fluorescence threshold around 20/30, and adjust the PI voltage to visualize the G0/G1 and G2/M populations on the PI-A axis at 50 and 100, respectively. The G2/M peak exhibits twice as much DNA per cell.
    d. Define different cell cycle phases using markers set within the analysis program (Figure 4D).

Note: For cell cycles analysis experiments, it is recommended using Flow Cytometers characterized by low pressure, such as the BD FACSCanto II Cytometer, and operate at low flow rate

during the acquisition. This improves resolution of cell distribution across the different phases of the cycle and yields better coefficient of variation (CV) values.

⚠ CRITICAL: Quickly acquiring samples is crucial to prevent chromosome dispersion in mitotic cells. Ensure the quality of the RNase used in sample preparation, as PI binds RNA and may cause incorrect measurements. Maintain consistent sample quality and compare samples with similar cell numbers, as these parameters affect measurement accuracy. Accurate interpretation of DNA histogram plots requires a minimum of 10,000 events for analysis.

Assessment of reversible cell states through *ex vivo* repopulation of FACS isolated cell fractions

Timing: 2 h 30 min

This procedure assesses the capacity of sorted cell fractions to repopulate the naïve distribution of CRIPTO expression levels, thereby indicating the presence of reversible cell states.

26. Centrifuge isolated CRIPTO positive and CRIPTO negative cell fractions at 400 × *g* for 4 min.
27. Resuspend cell pellets in low serum (1% goat serum-PBS 1×).
28. Split in two tubes each sample.
29. Incubate the cells in 1.5 mL tubes on a rotating wheel for 1 h at 25°C–26°C.
30. Re-stain the cells by adding 1:10 v/v anti-CRIPTO-APC antibody for 1 h at 25°C-26°C or leave them untreated.
31. Re-analyze the sorted samples using BD FACSAria III cell sorter, employing the same gating strategy and fluorescence intensity thresholds used during cell sorting to monitor CRIPTO protein level fluctuations.

Note: Both CRIPTO positive and CRIPTO negative cell fractions are re-stained to assess their ability to revert to a naïve state.[1,6] Unstained cell fractions are used as control: absence of fluorescence signals in CRIPTO negative cell fraction indicates that naïve state restoration depends on intracellular trafficking, while in CRIPTO positive cells indicates shedding of the protein/antibody complex (derived from the first staining) from the cell membrane rather than internalization.[7]

Additional Treatments: post-sorting, cells may undergo various treatments before re-staining, such as incubation at different temperatures affecting protein trafficking or the use of small molecules and inhibitors. For detailed information refer to.[1]

Cells can be cultured post-sorting and analyzed by FACS at different time points to monitor protein expression over time. The behavior of plated cells may differ from non-plated cells.

EXPECTED OUTCOMES

The heterogeneity of CRIPTO surface expression and its fluctuations reveal specific cellular mechanisms and activities within the activated MuSC population, as reported.[1] Flow cytometry technology has unequivocally supported the study and validated the *in vitro* data. By employing a rigorous gating strategy and specific sorting controls, comparisons across different experiments are anticipated. Consistent recovery protocols applied to various mouse samples ensure reliable and reproducible results. Additionally, this enables the analysis of time-course experiments of skeletal muscle regeneration. This protocol allows for the characterization of MuSC fractions identified based on different levels of CRIPTO expression, utilizing the sensitivity of flow cytometric measurements. Specifically, correlations with mean values of the side scatter (SSC) parameter, routinely monitored during sample acquisition for morphological evaluations, delineate differences in cellular activation

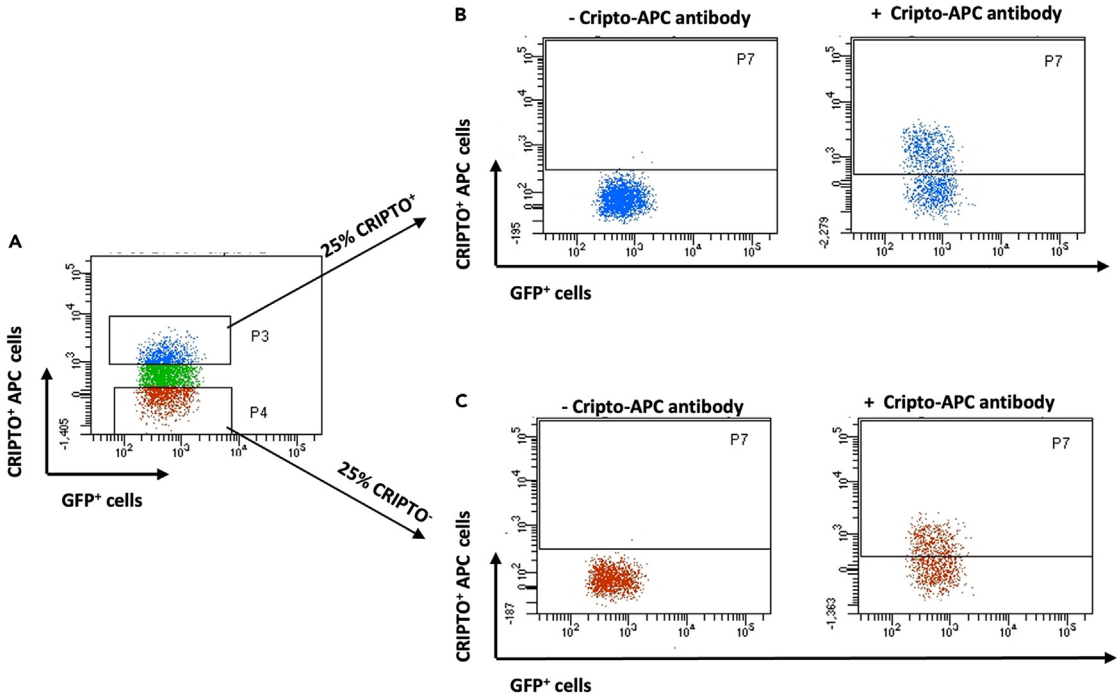

Figure 5. Repopulation assay

(A) Representative dot plot illustrating the fluorescence intensity of CRIPTO (anti-CRIPTO-APC antibody) vs. GFP, depicting the gating strategy for isolating CRIPTO-positive and CRIPTO-negative fractions of MuSCs, each comprising 25% of the total population.

(B and C) Representative dot plots displaying post-sorting cell fractions after reconstitution of naive CRIPTO expression levels, depicting the fluorescence intensity of anti-CRIPTO-APC antibody versus GFP. The plots delineate untreated and re-stained CRIPTO-positive (B) and CRIPTO-negative (C) MuSC fractions, representing the cells used in the repopulation assay.

states associated with cellular complexity. Similarly, other fluorescent markers, such as the GFP (Pax7) reporter used in this study, can be used for linear correlation with mean fluorescence intensity. These correlations may provide valuable insights into cellular activation, commitment, and self-renewal. In particular, our analysis aims to provide insights into the dynamics of cell surface membrane protein expression, distinguishing between micro- and macro-heterogeneity. Specifically, while micro-heterogeneity appears as a continuous distribution resembling a single peak, macro-heterogeneity is revealed as multiple peaks corresponding to different cell subpopulations. Furthermore, we aim to evaluate the capacity of isolated cell fractions or cellular subpopulations to revert to the naive state (reversible cell fraction) or remain stable (stable cell fraction) (Figure 5). Overall, comprehensive data on CRIPTO expression dynamics are expected, shedding light on cellular heterogeneity and functional properties within the population.

QUANTIFICATION AND STATISTICAL ANALYSIS

The flow cytometry data, processing and analysis, are conducted with BD FACSDiva software (v8.0.1) BD Biosciences. Statistical analysis is conducted with a minimum of three independent biological replicates for each experiment. Statistical significance between two groups was determined using unpaired Student's t-tests. Criteria for data inclusion and exclusion were applied consistently across all analyses.

LIMITATIONS

Regular and appropriate instrument maintenance helps minimize technical issues that could limit experimental reproducibility. The successful execution of flow cytometry analysis relies on the proper functioning of the instrument. Calibration is essential to verify laser alignment and establish optimal photomultiplier tubes (PMTs) baselines voltage for fluorescence detection in the samples.

Environmental factors also impact cytometers stability and performance. Room temperature regulation (25°C–26°C) is particularly important for cell sorting instruments like the BD FACS Aria III. High temperatures can destabilize the cell stream, affecting the drop delay, post-sorting recovery, and cell viability.

Samples derived from different mice may exhibit slight inter-subject variations in cell appearance on the FSC-A/SSC-A dot plot. Based on these sample characteristics, minor adjustments to baseline PMT voltages may be necessary to ensure the target cell population is centered within the correct gate.

High-quality antibodies and antigen specificity are essential. Poor-quality or low-purity antibodies may contain unconjugated fluorophores, leading to increased background signal, particularly problematic when detecting low percentages of positive cells for the antigen of interest.

TROUBLESHOOTING

Problem 1

A high proportion of cells are dead after cell sorting, and the number of cells recovered is lower than the number initially detected by BD FACSAria III. Referred to step 18.

Potential solution

It is crucial to start with a high-quality sample, as the cell death after sorting can affect approximately 20% of the recovered cells. During MuSCs isolation procedures, prioritize working quickly while minimizing interruptions, especially for steps performed at 25°C-26°C. For all other steps, maintain the cells on ice to minimize temperature fluctuations and ensure optimal viability.

Monitor the instrument during the sorting experiment to optimize recovery. If necessary, adjust the sheath fluid stream by regulating the amplitude and frequency to ensure the formation of uniform droplets.

Problem 2

During cell sorting, the instrument frequently clogs or the stream becomes unstable. Referred to step 18.

Potential solution

The sudden closure of the stream and its instability affect the sorting performance and the purity of the sorted population. Poor sample quality, particularly the presence of aggregates, can clog the microfluidic channels within the instrument. Before sorting, pipette the sample up and down to ensure a homogenous cell suspension. Visually inspect the sample and remove any visible debris that could clog the system. If necessary, use a new FACS filter to minimize clogging.

Avoid excess air bubbles before running it through the instrument.

Before re-starting, after stream closing, proceed as follows.

- Access "cytometer menu"
- Select "cleaning mode"
- Apply "sample line backflush" to clear the intake capillary
- Apply "clean flow cell" to clear the counting chamber.
- If necessary, sonicate the nozzle for 60 s.

Problem 3

The expression level of cell surface target protein is extremely variable and lower than usual. Referred to steps 12-15.

Potential solution

Inappropriate PMT voltage settings can affect signal intensity and resolution, leading to inaccurate data.

An improperly set negative threshold using the isotype control can result in false-positive events.

Variation in antibody quality, including using a new batch, can affect staining efficiency and data consistency.

Variations in marker expression can occur due to several factors affecting sample conditions, including temperature fluctuations, reagent quality, and incubation time variability.

Problem 4

Non-specific antibodies signal in FACS analysis when using antibodies targeting different epitopes or proteins. Referred to steps 12–15.

Potential solution

Perform antibody titration to determine the optimal concentration for specific staining with minimal background. Evaluate the necessity of blocking and washing steps to reduce non-specific binding while minimizing cell loss. To ensure specific staining and minimize background fluorescence, adhere to the recommended antibody incubation time provided by the manufacturer. Exceeding this time can lead to non-specific antibody binding, potentially resulting in elevated background and masking or creating false positives, particularly for antigens expressed at low levels.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact Ombretta Guardiola (ombretta.guardiola@igb.cnr.it).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact Laura Pisapia (laura.pisapia@igb.cnr.it).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This paper does not report original code.

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AUTHOR CONTRIBUTIONS

L.P. and O.G. conceptualized and supervised the project, performed the experiments, and wrote the article. V.M. and G.A. provided technical support. G.M. supervised the project and reviewed and edited of the article.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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