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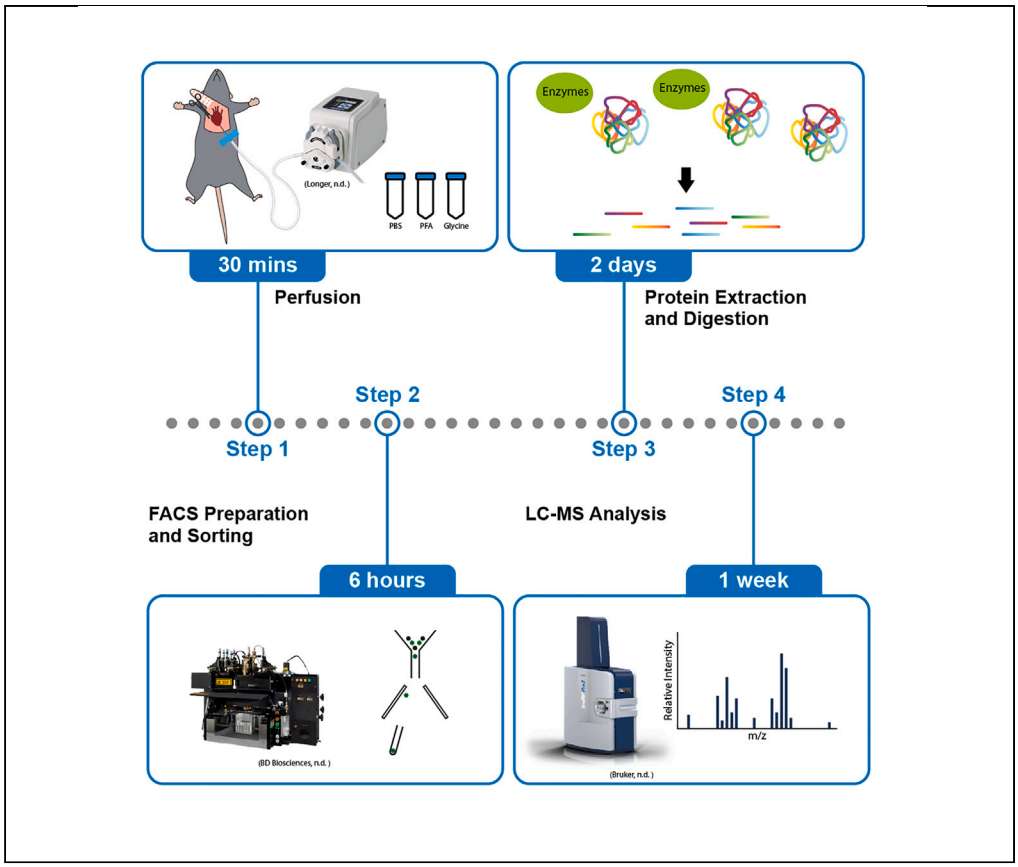
Protocol

Protocol for low-input proteomic analysis of *in situ* fixed adult murine muscle stem cells



Studying skeletal muscle stem cells (MuSCs) quiescence is challenging as they quickly activate within hours of isolation from muscle. Here, we present a protocol to disassociate and characterize fixed peptides from quiescent MuSCs using trapped ion-mobility time-of-flight mass spectrometry (MS). We describe steps for mouse perfusion, fluorescence-activated cell sorting preparation and sorting, protein extraction, digestion, and liquid chromatography MS analysis. This protocol can be applied to other less-abundant somatic stem cell types using mouse lines with a reporter.

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

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Highlights

Optimized protocol for low-input, high-throughput MS-based protein analysis

Detailed procedure for preparing peptide samples from *in situ* fixed muscle stem cells

Detailed parameter settings for identifying proteins by label-free quantitative MS

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Protocol for low-input proteomic analysis of *in situ* fixed adult murine muscle stem cells

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SUMMARY

Studying skeletal muscle stem cells (MuSCs) quiescence is challenging as they quickly activate within hours of isolation from muscle. Here, we present a protocol to disassociate and characterize fixed peptides from quiescent MuSCs using trapped ion-mobility time-of-flight mass spectrometry (MS). We describe steps for mouse perfusion, fluorescence-activated cell sorting preparation and sorting, protein extraction, digestion, and liquid chromatography MS analysis. This protocol can be applied to other less-abundant somatic stem cell types using mouse lines with a reporter.

For complete details on the use and execution of this protocol, please refer to Zeng et al. (2022, 2023).[1,2]

BEFORE YOU BEGIN

The protocol below describes the procedure of mouse anesthesia, perfusion, MuSC isolation[3] and the follow-up proteomic analysis using the transgenic mouse line: $Pax7^{CreERT2/+}$::$ROSA26^{YFP/+}$. Other reporter mouse lines which enable specific labeling of MuSCs (e.g., Pax7nGFP) can also be used. The age of mice used should be older than 2 months of age. There is no sex requirement.

To preserve the signature of quiescent MuSCs, we performed optimized paraformaldehyde (PFA)-perfusion as previously described.[4,5]

Institutional permissions

All experiments were approved by the HKUST Animal Ethics Committee.

Preparation of reagents for perfusion and protein precipitation

Timing: 2 h

Refer to Materials and Equipment for the buffer recipes.

1. Prepare 50 mL of acetone.

a. Pre-cool at –20°C for more than 1 day.
2. Prepare RIPA lysis buffer.
3. Prepare 1× PBS.
   a. Filter and pre-cool at 4°C.
   b. 30 mL is required for 1 mouse.
4. Prepare 2 M glycine.
   a. Prepare and filter, pre-cool at 4°C.
   b. 30 mL for 1 mouse.
5. Prepare 0.5% PFA.
   a. Prepare just before use.
   b. Pre-cool at 4°C.
   c. 30 mL is required for 1 mouse.
6. Prepare Wash Medium
   a. Store at 4°C for up to 1 month.
   b. 200 mL for 1 mouse.
7. Prepare Dissociation Buffer.
   a. Prepare just before use.
   b. 10 mL for 1 mouse.
8. Prepare Stock Collagenase II Solution.
   a. Store at –20°C for up to 1 year.
   b. 1 mL is required for 1 mouse.
9. Prepare Stock Dispase Solution.
   a. Store at −20°C for up to 1 year.
   b. 1 mL is required for 1 mouse.
10. Prepare pentobarbitone.
    a. Store at 4°C for up to 1 month.
11. Prepare the perfusion stage.
    a. Prepare an ice bucket to hold the 1× PBS, 0.5% PFA, and 2 M Glycine.
    b. Connect the infusion set to the precise peristaltic pump and set the speed of the pump to 70 rpm.
    c. Set up the foam plate and collection box.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
32% Paraformaldehyde (PFA) aqueous solution, EM grade	Electron Microscopy Sciences	Catalog #: 15714
Acetonitrile (ACN)	Honeywell	Catalog #: 34967
Acetone puriss. p.a., ACS reagent, ≥99.5% (GC)	Honeywell	Catalog #: 32201
Collagenase, type 2	Worthington Biochemical	Catalog #: LS004177
Dispase II, powder	Thermo Fisher Scientific	Catalog #: 17105041
Dithiothreitol (DTT)	USB Corporation	Catalog #: 3483-12-3
Ethanol absolute	VWR Chemicals	Catalog #: 20821.330
Formic acid	Thermo Fisher Scientific	Catalog #: 85178
Glycine, for electrophoresis, ≥99%	Sigma-Aldrich	Catalog #: G8898
Ham's F-10 nutrient mix	Thermo Fisher Scientific	Catalog #: 11550043
Horse serum (HS)	Invitrogen	Catalog #: 16050114
Iodoacetamide (IAA)	Sigma-Aldrich	Catalog #: I1149-25G
Ambion molecular grade ethylenediaminetetraacetic acid (EDTA) (0.5 M), pH 8.0	Thermo Fisher Scientific	Catalog #: AM9260G

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Pentobarbital sodium (Dorminal 20% inj.)	Alfamedic	Catalog #: 013003
Phosphate-buffered saline (PBS)	Sigma-Aldrich	Catalog #: P3813
Penicillin-Streptomycin (P/S) (10,000 U/mL)	Thermo Fisher Scientific	Catalog #: 15140122
Sodium chloride (NaCl)	Sigma-Aldrich	Catalog #: 31434
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich	Catalog #: L3771-500G
Sodium deoxycholate	Sigma-Aldrich	Catalog #: 89904
Trifluoroacetic acid	Sigma-Aldrich	Catalog #: T6508
Triton X-100, laboratory grade	Sigma-Aldrich	Catalog #: X100-500ML
Trizma base, primary standard and buffer, >=99.9% (titration), crystalline	Sigma-Aldrich	Catalog #: T1503-100G
UltraPure DNase/RNase-free distilled water	Thermo Fisher Scientific	Catalog #: 10977015
Critical commercial assays		
iST kit 96×	PreOmics	Catalog #: P.O.00027
BCA protein assay kit	Thermo Fisher Scientific	Catalog #: 23225
Experimental models: Organisms/strains		
Mouse: ROSA26YFP/YFP (male, 2–4 months old)	JAX	JAX: 006148
Software and algorithms		
PEAKS software (Version: Xpro)	Bioinformatics Solutions, Inc.	N/A
Other		
20 mL syringe	Terumo	Catalog #: SS+20L
40 μm cell strainer	SPL Life Sciences	Catalog #: 93040
Acid-resistant refrigerated CentriVap vacuum concentrators	Labconco	Catalog #: 7310034
BD Influx cell sorter	BD Biosciences	N/A
CentriVap –105°C cold trap	Labconco	Catalog #: 7385035
C18 column	IonOpticks	Catalog #: AUR2-25075C18A-CSI
Disposable needle 20 G × $1^{1/2}$ inch	Terumo	Catalog #: NN+2038R
Eppendorf centrifuge 5427R	Eppendorf	Catalog #: 5427R
Eppendorf centrifuge 5804R	Eppendorf	Catalog #: 5804R
50 mL high clarity PP centrifuge tube	Falcon	Catalog #: 352070
Filter upper cup, PES 0.22 μm, 500 mL, 75 mm	Jet Bio-filtration	Catalog #: FPE214150
MD 4C NT chemistry diaphragm vacuum pump	Vacuubrand	Catalog #: 7393001
Precise peristaltic pump	Longer Precision Pump	Catalog #: BT100-2J
Round-bottom polypropylene tube, 5 mL	Falcon	Catalog #: 352063
Round-bottom polystyrene test tube, with cell strainer snap cap, 5 mL	Falcon	Catalog #: 352235
Syringe filter, Minisart high flow, 0.22 μm, PES membrane, sterile, 28 mm	Sartorius	Catalog #: 1209Z85
SURFLO winged infusion set	Terumo	Catalog #: SV*23NL
Shaking water bath	Memmert	Catalog #: WNB 22
timsTOF Pro mass spectrometer or other equivalent low-input mass spectrometer	Bruker or others	N/A

MATERIALS AND EQUIPMENT

0.5% PFA (for 1 mouse)	
Reagent	Amount
32% PFA	468.75 μL
1× PBS	29.5 mL
Total	**30 mL**
Keep at 4°C, prepare just before use.	

⚠ CRITICAL: PFA is water-soluble and should always be used with adequate ventilation, preferably in a fume hood. Eye and skin exposure should be avoided. Follow the materials safety data sheet when handling PFA.

2 M Glycine (for ~16 mice)	
Reagent	Amount
Glycine	7.5 g
1× PBS	500 mL
Total	**500 mL**
Store at 4°C for up to 1 month.	

Note: Filter reagent through 0.22 μm filter before use.

20 mg/mL Pentobarbitone (for ~25 mice)	
Reagent	Amount
Pentobarbital Sodium (200 mg/mL)	0.5 mL
Sterile 0.9% NaCl	4.5 mL
Total	**5 mL**
Store at 4°C for up to 1 month.	

Stock Collagenase II Solution (for 1 mouse)	
Reagent	Amount
Collagenase II	3000 U
1× PBS	1 mL
Total	**3000 U/mL**
Store at −20°C for up to 1 year.	

Stock Dispase Solution (for 1 mouse)	
Reagent	Amount
Dispase	33 U
1× PBS	1 mL
Total	**33 U/mL**
Store at −20°C for up to 1 year.	

Wash medium (for ~2 mice)		
Reagent	Final concentration	Amount
Ham's F-10 Nutrient Mix	N/A	450 mL
HS	10%	50 mL
P/S	1%	5 mL
Total	**N/A**	**500 mL**
Store at 4°C for up to 1 month.		

Note: Filter HS with a 0.22 μm filter before adding it to the wash medium.

Muscle Dissociation Buffer (for 1 mouse)		
Reagent	Final concentration	Amount
Wash Medium	N/A	10 mL
Collagenase II	2000 U/mL	N/A
Total	**2000 U/mL**	**10 mL**
Keep at 4°C, prepare just before use.		

RIPA lysis buffer (with 1 mM EDTA and 2% SDS)		
Reagent	Final concentration	Amount
Tris-HCl (1 M, pH 7.4)	50 mM	25 mL
NaCl	150 mM	4.383 g
Sodium deoxycholate	1.5%	7.5 g
Triton X-100	1%	5 mL
EDTA (0.5 M)	1 mM	1 mL
SDS	2%	10 g
Water	N/A	470 mL
Total	**N/A**	**500 mL**
Store at 4°C.		

STEP-BY-STEP METHOD DETAILS

Mouse perfusion

Timing: ~30 min per mouse

Perfusion allows the PFA to pass through the circulatory system to fix MuSCs *in situ*.

1. Prepare 30 mL of 1× PBS, 30 mL of 0.5% PFA, and 30 mL of 2 M Glycine in 50 mL falcon tubes with labels for each mouse and place them on ice.
2. Set up the perfusion pump at 70 rpm (~5 mL/min) and the dissection stage.
3. Anesthetize the mouse with 20 mg/mL sodium pentobarbital via intraperitoneal injection.

Note: Suggested dosage for mice anesthetization

Body weight (g)	Sodium pentobarbital volume (mL)
20	0.05–0.1
30	0.07–0.13
40	0.1–0.18
50	0.12–0.2

Note: Pinch the mouse by the toe to check whether it is fully anesthetized. Provide an extra dose of anesthetization reagent if needed.

4. After the mouse is anesthetized, lay the mouse on the dissection stage in the supine position and immobilize with surgical tape/needles. Open the chest and expose the heart for perfusion.
5. Open the pump to drain the air from the needle with 1× PBS and stop. Cut on the right atrium for fluid removal. Pierce the heart in the left ventricle with the butterfly needle. (Figure 1)
6. Perfuse the mouse with cold 1× PBS for 5 min, then 0.5% PFA for 5 min, and finally with 2 M Glycine for 5 min to quench formaldehyde.

Note: Pause the pump during solution change. Prevent air from entering the circulatory system.

FACS preparation and cell sorting

Timing: Muscle dissociation: ~4 h; FACS sorting: ~1 h per mouse

The detailed protocol for the preparation of single-cell suspension from unfixed tissue was previously published by Liu et al., 2015. We doubled the collagenase II amount in dissociation buffer for fixed tissue.

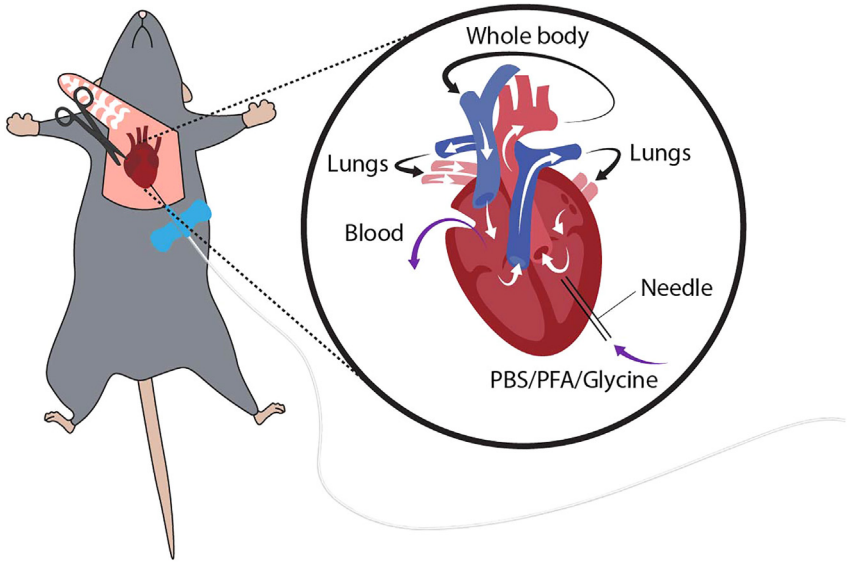

Figure 1. Graphical illustration of the fluid direction in the heart during perfusion

7. Prepare a 10 cm glass Petri dish with 10 mL cold wash medium for each mouse.
8. Dissect the hindlimb muscles and place them into the Petri dish with wash medium.
9. Transfer the muscle to the lid of the Petri dish and mince the muscles into fine pieces (~1 mm^3) in 10 mL dissociation buffer.
10. Transfer the minced muscle pieces with dissociation buffer to a 50 mL falcon tube.
11. Seal the tube with Parafilm and incubate in a 37°C shaking water bath for 90 min at 65 rpm.
12. Top up the tube to 50 mL with fresh cold wash medium.
13. Centrifuge the tissue suspension at 500 g for 10 min at 4°C. Aspirate the supernatant until only 20 mL remains in the tube.
14. Add 1 mL of stock Collagenase II solution and 1 mL of stock Dispase solution to the tube and resuspend the pellet.
15. Seal the tube with Parafilm and incubate in a 37°C shaking water bath for 30 min at 65 rpm.
16. After digestion, use a 20 mL syringe and a 20-gauge needle to aspirate and eject the muscle suspension 10 times.

Note: Remove the undigested pieces that may clog the needle. Eject the muscle suspension towards the wall of the tube to avoid foaming.

17. Place a 40 μm nylon cell strainer onto a new 50 mL falcon tube. Transfer the cell suspension to the new 50 mL falcon tube through the cell strainer.
18. Add 20 mL of cold wash medium to the original tube. Swirl to rinse and transfer it through the same cell strainer.
19. Top up the tube to 50 mL with fresh cold wash medium.
20. Centrifuge the tube at 500 g for 10 min at 4°C. Remove all of the supernatant without disturbing the cell pellet.
21. Resuspend the cell pellet in 1 mL of wash medium. Transfer the cell suspension to a fresh 5 mL FACS tube through the filter cap.
22. Rinse the 50 mL falcon tube with 500 μL cold wash medium and transfer it to the FACS tube through the filter cap.
23. Set up the cell sorter following the manufacturer's specifications with a 70 μm nozzle.

Note: Place the cell suspension on ice while waiting.

24. Collect the singlet YFP$^+$ population at room temperature (25°C) into 1 mL of 1× PBS in a FACS collection tube.

Note: The sorting plot refers to our previously published STAR Protocol.[4]

Cell lysis and de-crosslinking

Timing: ~1 day

25. Centrifuge the cell suspension at 5000 g, 4°C for 10 min.
26. Aspirate the supernatant carefully. Do not disturb the cell pellet.

Pause point: The cell pellet can be stored at −80°C by snap-freezing in liquid nitrogen.

27. Add 100 μL of RIPA buffer (containing 2% SDS) and mix by pipetting up and down.
28. Incubate at 70°C for 2 h to de-crosslink the proteins.
29. Measure the protein concentration using the BCA assay.
30. Cool the cell lysate on ice. Add 400 μL of pre-cooled acetone to the lysate. Mix by pipetting up and down.
31. Store the cell lysate overnight (for 12 h) at −20°C.
32. Centrifuge at 15000 g, 4°C for 30 min and discard the supernatant.
33. Wash protein pellet with 500 μL of pre-cooled (at –20°C) acetone, then 500 μL of 70% pre-cooled ethanol, and finally with 500 μL of pre-cooled acetone. (Centrifuge at 15000 g, 4°C for 5 min after each wash).
34. Dry the protein pellet using the vacuum concentrator at 25°C for 5 min.

Protein digestion and de-salting

Timing: ~3 h

Sample preparation for bottom-up mass-spectrometry was carried out on the PreOmics system using the iST Sample Preparation Kit, which includes lysis denaturation, reduction, alkylation, Lys-C and trypsin digestion and peptide purification. We altered the lysis temperature to 83°C. Below is a simplified protocol based on the PreON system. The CAPITAL reagents are provided in the iST kit.

35. Add 50 μL of LYSE to 1–100 μg of protein sample, and then place the sample in the HEATING BLOCK (set to 83°C; 1000 rpm; 10 min).
36. Add 210 μL of RESUSPEND to DIGEST (1 tube for 4 reactions), pipette up and down.
37. Add 50 μL of DIGEST to sample tube and rotate at 37°C, 500 rpm for 1 h.
38. Add 100 μL of STOP to sample tube (precipitation may occur), shake at room temperature (25°C), 500 rpm for 1 min.
39. Transfer the sample solution mixture to the CARTRIDGE. Spin the CARTRIDGE at 2250 rcf for 1–3 min).

Note: Adjust the time if needed to ensure complete flow-through.

40. Add 200 μL of WASH 1 to the CARTRIDGE. Spin the CARTRIDGE at 2250 rcf for 1–3 min.
41. Add 200 μL of WASH 2 to the CARTRIDGE. Spin the CARTRIDGE at 2250 rcf for 1–3 min.
42. Move the CARTRIDGE to the 1.5 mL collection tube.
43. Add 100 μL of ELUTE and spin the CARTRIDGE at 2250 rcf for 1–3 min.
44. Repeat step 43 and keep the flow-through in the collection tube.
45. Dry the eluent from the purification column of the iST kit in the Vacuum Concentrator at 37°C for 2 h and reconstitute in 0.1% formic acid (98% H_2O, 2% acetonitrile (ACN) and 0.1% FA).

Alternatives: Other methods of protein digestion can be used. We used trypsin as an alternative. Steps 46–55 describe the detailed procedure of protein digestion and de-salting using trypsin and Pierce C18 Spin Tips.

46. Resuspend the pellet with 10 μL of UA buffer (8 M urea, 0.1 M Tris-HCl, pH 8.5).
47. Add 2.25 μL of 10 mM DTT (final concentration 2 mM), vortex and spin down. Keep at 30°C for 1.5 h.

⚠ CRITICAL: DTT is under the category of chemical hazards, please put on appropriate personal protective equipment and ensure proper disposal into designated chemical waste containers.

48. Add 2.83 μL of 50 mM Iodoacetamide (IAA) buffer (50 mM IAA in 50 mM Ammonium bicarbonate buffer) (final concentration of IAA is 10 mM), vortex and spin down. Keep at room temperature (25°C) and protect from light for 40 min.

⚠ CRITICAL: IAA is toxic if swallowed and can cause severe skin burns and eyes damage. Put on appropriate personal protective equipment and use only with adequate ventilation. Follow the materials safety data sheet when handling IAA.

49. Add 90 μL of 50 mM NH_4HCO_3 to the mixture; ensure the final concentration of urea is 0.7 M.
50. Add trypsin in 1:50 w/w, digest at 37°C for 4 h.
51. Add the same amount of trypsin as step 50 and digest overnight (for 12 h) at 37°C.
52. Add a sufficient volume of 10% trifluoroacetic acid (TFA) to cease digestion. (The final TFA concentration will be 0.4%).
53. Concentrate the sample to 20 μL using the vacuum concentrator. If the volume is less than 20 μL or dried out, reconstitute the samples in 0.1% TFA for subsequent desalting.
54. Desalt the samples using Pierce C18 spin tips.
55. Dry the eluent from the column in the Vacuum Concentrator at 37°C for 2 h and reconstitute in 0.1% formic acid (98% Milli-Q water, 2% ACN and 0.1% formic acid (FA)).

Mass-spectrometry settings

For a detailed description of the label-free quantitative mass-spectrometry, please view Zhu et al.[6,7]

56. Inject 200 ng of peptides into the IonOpticks 25 cm Aurora Series emitter column with CSI (1.6 μm C18). Analyze using the Bruker nanoElute coupled to the timsTOF Pro mass spectrometer.
57. Elute the peptides from the column using mobile phases A (98% Milli-Q water, 2% ACN with 0.1% FA) and B (100% ACN with 0.1% FA) while keeping the column at a constant temperature of 50°C.
58. Set LC parameters as follows:
    a. LC Parameters.

Elution mode	One column separation
Separation column	IonOpticks 25 cm
Column temperature	50°C

b. LC gradient.

Time (min)	Composition (%B)	Flow rate (μL/min)
0.00	2.0	0.30
0.50	5.0	0.30
27.00	30.0	0.30

(*Continued on next page*)

Continued		
Time (min)	Composition (%B)	Flow rate (μL/min)
27.50	95.0	0.30
28.00	95.0	0.30
28.10	2.0	0.30
30.00	2.0	0.30

59. timsTOF settings.

a. General settings.

Ion mobility Mode	Enable tims	
MS Data reduction	Yes	
Mass Spectra Peak Detection	Use Maximum Intensity	
Absolute Threshold	10	
Absolute Threshold (per 100 ms Accu time)	10	
Mobilogram Peak Detection		
Intensity Threshold	Absolute	5000
TIMS	Enable	
Ion Polarity	Positive	
Scan mode	PASEF	
Scan range		
Mass	100–1700 m/z	
1/k0	0.85–1.3 (V x s/cm^2)	
Rolling average	10×	

b. tims settings.

imeX	
Resolution	Custom
1/k0 Stat	0.85 (V x s/cm^2)
1/k0 End	1.3 (V x s/cm^2)
Ramp Time	100.0 ms
Spectra Rate	9.52 Hz
Advance Parameter	
Lock Accumulation to mobility range	Lock Duty Cycle to 100%
Cycle time	100 ms

c. Source.

Source	CaptiveSpray
Capillary	1400 V/ 1168 nA
Dry Gas	3.0 L/min
Dry Temp	180°C

d. Tune.

Funnel 1 RF	300.0 Vpp
Funnel 2 RF	200.0 Vpp
isCID Energy	0.0 eV
Multipole RF	200.0 Vpp
Deflection Delta	70.0 V
Quadrupole	
Ion Energy	5.0 eV
Low Mass	200.00 m/z

Collision Cell	
Collision Energy	10.0 eV
Collision RF	1500.0 Vpp
Transfer Time	60.0 µs
Pre Pulse Storage	12.0 µs
Delta Values	
Delta 1	-20.0 V
Delta 2	-160.0 V
Delta 3	110 V
Delta 4	110 V
Delta 5	0.0 V
Delta 6	55.0 V
Funnel 1 RF	350.0 Vpp
Collision Cell	300.0 V

e. MS/MS (PASEF).

Isolation Width			
Mass m/z	690	800	
Width m/z	2.00	2.99	
Collision Energy			
1/k0 V*s/cm^2	0.85	1.30	
Energy eV	27.00	45.00	
Precursor Ions			
MS Repetitions	1×		
Cycle Overlap	1		
Scheduling			
Intensities	2 × 10^5	1 × 10^5	0
Repetitions	0	5	10
Mobilogram			
Summation Width	25 pts.		
Max No. of Peaks	3		
Active Exclusion			
Mass Width	0.015 m/z		
1/k0 Width	0.015 (V × s/cm^2)		
Precursor Ions			
No. of PASEF MS/MS scans	4		
Total cycle time	0.53 s		
Scheduling			
Target intensity	20000		
Intensity Threshold	2500		
Change Range	0–5		
Active Exclusion	Release after 0.4 min		
Reconsider Presursor, if current intens./Previous intens. 4.00			

Protein identification and quantification using PEAKS studio

Timing: 2–3 h per sample depending on the data size

This part describes the use of PEAKS Studio software (Version Xpro) for LC-MS/MS data analysis. We suggest using the newest version for discovery proteomics. Other software such as MaxQuant can also be used for data analysis. The database for searching is UniProt and the taxonomy is *Mus musculus* in this protocol.

60. Select "Sequence Database Search" in the Workflow Selection window.

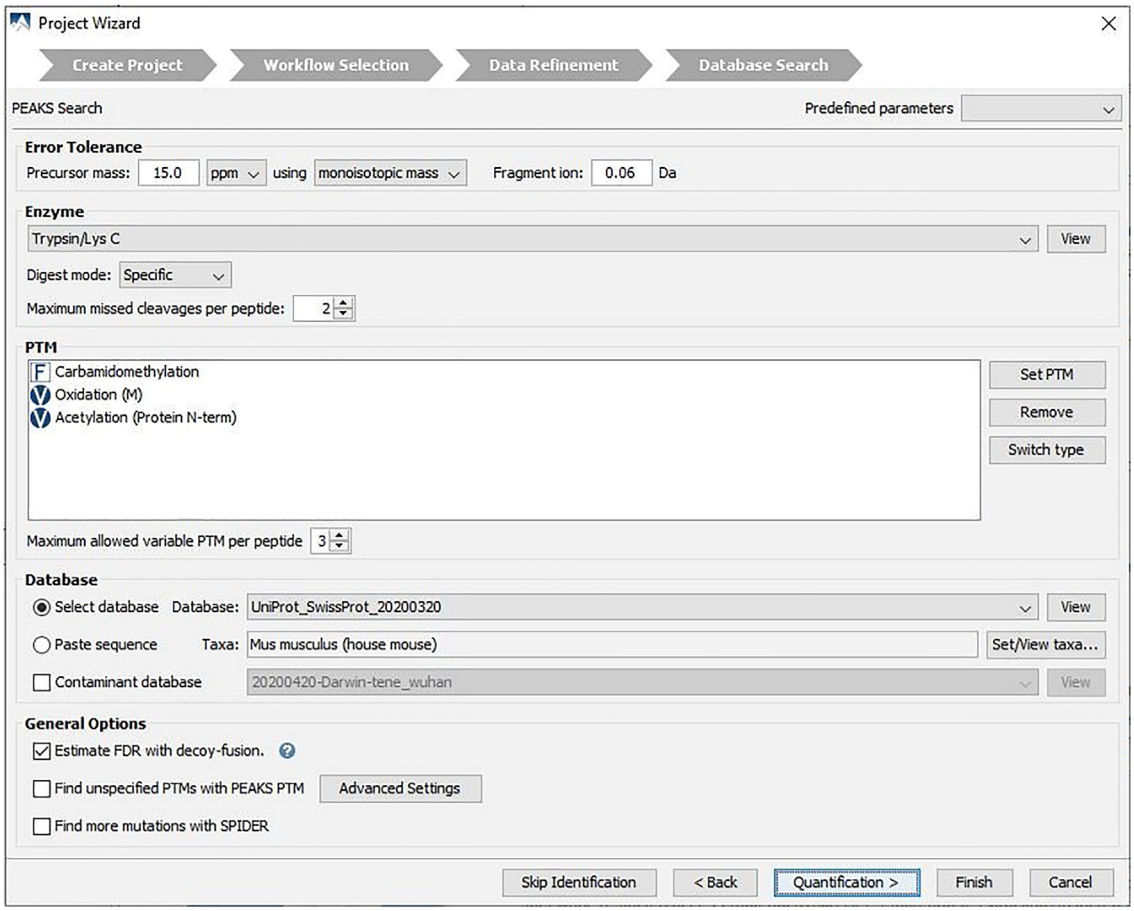

Figure 2. Settings of the database search

Note: We are using Sequence Database Search for our PASEF-DDA method. For DIA approach, use Spectral Library Search instead.

61. Data refinement.
    a. Select Precursor [DDA] – Mass only.
    b. Select "Associate feature with chimera scan [DDA].
    c. Select "Filter Features" – "Only keep features satisfying" – "Charge between 2 and 8".
62. Database search. (Figure 2)

The criteria of the protein search are related to the sample preparation process. Here we provide the settings corresponding to the iST kit.

63. Perform quantification and save results.

EXPECTED OUTCOMES

This protocol describes how to identify and quantify proteins from quiescent muscle stem cells. We are able to acquire 10 μg of protein from 100 - 300 k quiescent MuSCs isolated from one Pax7$^{CreERT2/+}$::ROSA26$^{YFP/+}$ mouse. The practice of perfusion techniques and fixation quality control is suggested to ensure stable output. The timsTOF system allows us to identify approximately 2000 proteins in one sample. This protocol also applies to samples that require fixation before analysis by LC-MS/MS at different time points, which is important for the study of stem cell quiescence and early activation.

LIMITATIONS

Perfusion using fixatives is essential to preserve the quiescence signature of muscle stem cells. However, operational problems, such as an incorrect angle and position of the needle into the heart, or a dislodged needle during perfusion can result in perfusion failure.

Protein diversity and abundance are limited in quiescent MuSCs. Due to technical limitations, less abundant proteins remain difficult to detect without enrichment.

This protocol only works on mouse lines with reporter proteins.

TROUBLESHOOTING

Problem 1

Incomplete fixation or over fixation (step 6).

Potential solution

Ensure the fluid comes out of the right atrium.

- Ensure the 0.5% PFA solution is freshly prepared and mixed well.
- Ensure the needle is inserted in the correct position (left ventricle) during the whole perfusion. Do not poke the needle too deep into the right ventricle.
- Ensure the flow is smooth and the tube is filled with PBS before starting (step 5). Pause the pump when changing the solution. Air in the blood vessels may affect perfusion.
- Ensure the mouse is alive before the perfusion begins. Blood coagulation will cause perfusion failure.

Note: The sorting plot for abnormal fixation refers to our previously published STAR Protocol.[4]

Problem 2

Low yield in fixed-sorted cell number (step 24).

Potential solution

- Ensure the muscle tissue is minced thoroughly in step 9. Large muscle pieces cannot be fully dissociated by enzymes and lead to clogging of the needles in step 16.
- Ensure the gating on the sorting plot selects the whole population. Fixed MuSCs have a smaller forward scatter (FSC) compared to freshly isolated MuSCs.
- Ensure the centrifugation is efficient. The pellet in steps 13 and 20 should remain intact after centrifugation and during aspiration.

Problem 3

Abnormal protein amount (step 29).

Potential solution

- The workflow is able to extract ~5–10 μg of protein from one mouse.
- Quantify the actual cell number after sorting.
- Ensure the cell pellet is intact after centrifugation (step 25).
- Ensure the assay used for quantifying the protein concentration is compatible with SDS.

Problem 4

Low outcomes after data analysis (step 63).

Potential solution

Adjust the de-crosslink conditions. Incomplete de-crosslink can result in low yield (step 28).

Problem 5

Abnormal chromatogram (step 58).

Potential solution

- Contamination of column may affect the MS to select the peaks from the samples for analysis which may change the chromatogram and reduce the protein ID number that can be detected.
- Remove detergent before protein digestion. Flush the column with a high percentage of organic solvent if there is contamination in the LC system (e.g., magic solution 25:25:25:25 MS/HPLC-grade H2O/ACN/MeOH/IPA).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Prof. Tom Cheung (tcheung@ust.hk).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze any datasets or code.

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AUTHOR CONTRIBUTIONS

Conceptualization, W. Zhang, W. Zeng, and T.H.C.; investigation, W. Zhang and P.S.W.; writing – original draft, W. Zhang; writing – review and editing, W. Zhang., W. Zeng., P.S.W., and T.H.C.; funding acquisition, T.H.C.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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