

Extraction and sequencing of single nuclei from murine skeletal muscles

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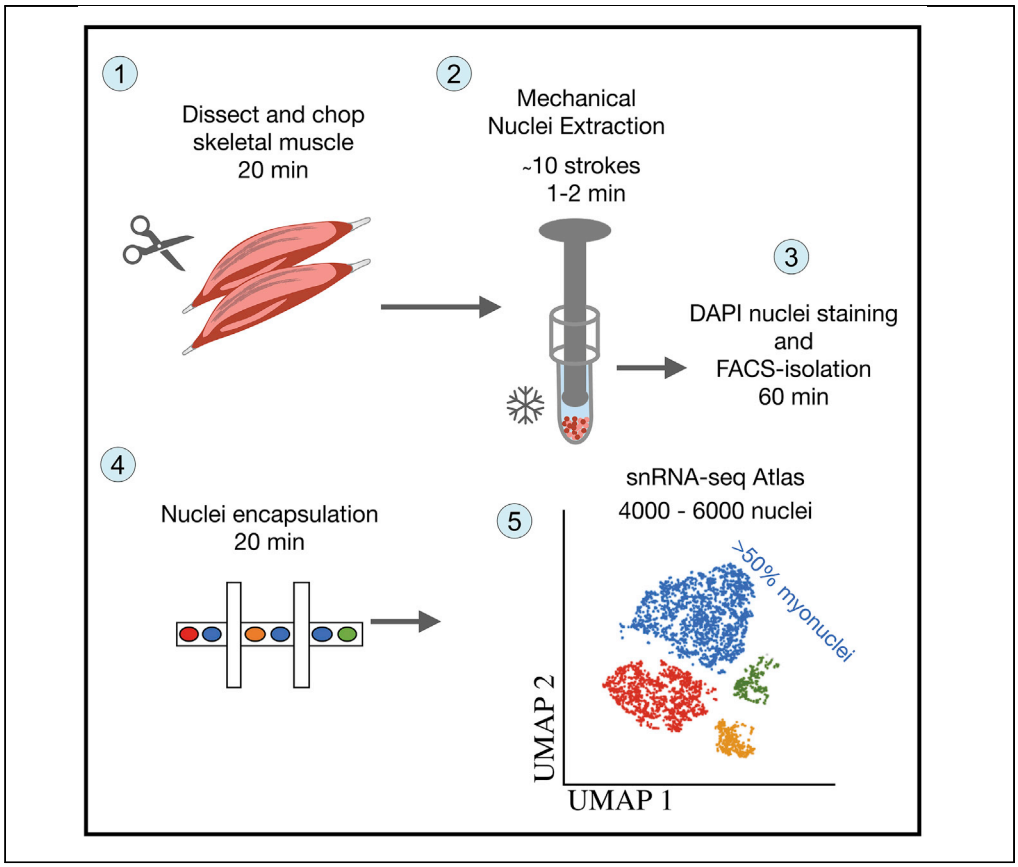
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Protocol

Extraction and sequencing of single nuclei from murine skeletal muscles



Single-nucleus RNA sequencing allows the profiling of gene expression in isolated nuclei. Here, we describe a step-by-step protocol optimized for adult mouse skeletal muscles. This protocol provides two main advantages compared to the widely used single-cell protocol. First, it allows us to sequence the myonuclei of the multinucleated myofibers. Second, it circumvents the cell-dissociation-induced transcriptional modifications.

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Highlights

snRNA-seq captures essentially all cell populations present in the skeletal muscle tissue

Can identify rare myonuclei populations, like neuromuscular and myotendinous myonuclei

snRNA-seq circumvents cell-dissociation-induced modifications on gene expression

In contrast to single-cell RNA-seq, snRNA-seq allows us to process snap frozen biopsies

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Extraction and sequencing of single nuclei from murine skeletal muscles

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SUMMARY

Single-nucleus RNA sequencing allows the profiling of gene expression in isolated nuclei. Here, we describe a step-by-step protocol optimized for adult mouse skeletal muscles. This protocol provides two main advantages compared to the widely used single-cell protocol. First, it allows us to sequence the myonuclei of the multinucleated myofibers. Second, it circumvents the cell-dissociation-induced transcriptional modifications.

For complete details on the use and execution of this protocol, please refer to Dos Santos et al. (2020) and Machado, Geara et al. (2021).

BEFORE YOU BEGIN

Cleaning and buffer preparation

Timing: 2–3 h

1. Cleaning up:
   a. Clean the Dounce homogenizer, the pestle and the dissection tools first with dH_2O, then with 70% EtOH and finally by dry heat sterilization at 160°C for 2 h.
   b. Clean the bench first with dH_2O to remove dust (main source of RNases), then with 70% EtOH and RNaseZAP™ solution or equivalent to eliminate RNase.

2. Buffer preparation (see complete recipes in the materials and equipment section):
   a. The preparation of the buffers and the nuclei extraction are carried out under a tissue culture hood. Buffers should be prepared fresh and kept on ice.
   b. Prepare 6 mL of lysis buffer per sample using RNase-free solutions (10 mM Tris-HCl, 10 mM NaCl, 3 mM $MgCl_2$, and 0.1% NonidetTM P40 in Nuclease-Free H_20).
   c. Prepare 15 mL of wash buffer per sample (PBS, 2% BSA).
   d. Make 3 mL of wash buffer per sample (PBS, 2% BSA) + RNase inhibitor by adding RNase inhibitor to a final concentration of 0.2 U/μL.
   e. Place the lysis and wash buffers on ice.

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Note: It is advised to prepare and label the tubes in advance, according to the following chart (tubes per sample):

i. 1× 2 mL tube
ii. 2× 50 mL conical tubes
iii. 2× 15 mL conical tubes
iv. 1× 1.5 mL DNA low-binding tube (e.g., Eppendorf DNA LoBind)

3. Pre-cool the centrifuges for 15 mL and 1.5 mL tubes at 4°C.
4. Place a 70 μm and a 40 μm cell strainer on two 50 mL conical tubes on ice. Rinse the cell strainers with 1 mL of wash buffer without RNase inhibitor. Discard the wash buffer.
5. Prepare a 15 mL of solution of 50% glycerol in water. Filter the solution with a 0.2 μm filter. Store the solution in a 2 mL tube at -20°C.
6. When using a new 10X Single Cell 3′ kit: reconstitute lyophilized the Template Switch Oligo from the 10x Genomics kit by adding 80 μL of low TE buffer. Vortex mix vigorously for 15 s and centrifuge briefly. Leave at 18°C–25°C for at least 30 min. After reconstitution, store at -80°C.
7. Just before starting the experiments, equilibrate at room temperature the 50% glycerol, the Template Switch Oligo, the Reducing Agent B and the Reverse Transcription (RT) Reagent from the 10×Genomics kit. Note: the Single cell 3′ Gel beads (included in the kit) have to be removed from -80°C and equilibrate at room temperature 30 min before loading the 10x Genomics chip.

Note: Accurate quantification of nuclei concentration is extremely important for 10X Genomics applications, and any mistake in counting can result in undesired outcome with either low or excessive number of encapsulated nuclei. In this protocol, we load the nuclei directly after Fluorescence-activated cell sorting (FACS) in the 10X Genomics machine without centrifugation. The nucleus concentration after FACS is expected to be constant when the same sorting parameters are applied. FACS machines often overestimate the number of nuclei so the actual concentration should be determined empirically using an automatic cell counter (we find this to be a more accurate method than manual counting). This quality test also allows fine-tuning the gating parameters of the cytometer that will be used the day of the experiment.

To FACS the nuclei, we used a BD FACSAria III with an 85 μm nozzle. The concentration of nuclei after FACS was approximately 250 nuclei/μL and we loaded 40 μL into the 10x Genomics Chip. Using a larger nozzle (e.g., 100 μm) will decrease the nuclei concentration to suboptimal levels and might not allow loading the nuclei directly after FACS into the chip. In contrast, using a smaller nozzle (70 μm) increases the concentration but also augments the pressure and the risk of damaging the nuclei.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
1M Tris-HCl pH 7.4	Sigma-Aldrich	Cat#T2194
5M NaCl	Sigma-Aldrich	Cat#59222C
1M $MgCl_2$	Sigma-Aldrich	Cat#M1028
NonidetTM P40	Sigma-Aldrich	Cat#74385
Nuclease-Free Water	Thermo Fischer Scientific	Cat#AM9932
Phosphate-buffered saline (PBS) with 10% bovine albumin	Sigma-Aldrich	Cat#A1595
RNase inhibitor	Roche	Cat#3335399001
4′, 6-Diamidino-2-phenylindole (DAPI)	Thermo Fischer Scientific	Cat#D1306
Glycerol	Merck Millipore	Cat#356352

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Low TE Buffer	Thermo Fischer Scientific	Cat#12090-015
QIAGEN Buffer EB	QIAGEN	Cat#19086
10% Tween 20	Bio-Rad	Cat#1662404
100% EtOH	Sigma-Aldrich	Cat#E7023
Critical commercial assays		
Chromium Controller & Next GEM Accessory Kit	10x Genomics	Cat#1000202
10X Single Cell 3′ kit, Chromium Next GEM Chip G Single Cell Kit, 16 rxns	10x Genomics	Cat#1000127
10X Single Cell 3′ kit, Chromium Next GEM Single Cell 3′ GEM, Library & Gel Bead Kit v3.1, 16 rxns	10x Genomics	Cat#1000121
DynabeadsTM MyOneTM SILANE	10x Genomics	Cat#2000048
Single Index Kit T Set A	10x Genomics	Cat#1000213
SPRIselect Reagent Kit	Beckman Coulter	Cat#B23318
Deposited data		
Raw and analyzed data	This protocol	GSE150065 and GSE163856
The script used for the Seurat analysis	Dos Santos et al., 2020	https://github.com/matthieudossantos/Single-nuclei-RNAseq-and-single-nuclei-ATACseq-script-for-Seurat/blob/master/script_single_nuclei_RNAseq.r
Experimental models: organisms/strains		
8–12-week-old C57Black6n female mice	Janvier Labs	n/a
Software and algorithms		
R studio	R Core Team	Version 4.0.3
Seurat	Stuart et al., 2019	Version 3.0.2
Cell Ranger	10x Genomics	Version 3.0.2
STAR	Dobin et al., 2013	Version 2.4.0
Other		
Countess™ II Automated Cell Counter	Thermo Fischer Scientific	Cat#A27978
15 mL Dounce homogenizer, loose pestle (pestle A)	Sigma-Aldrich	Cat#D9938
Falcon Cell strainer 70 µm	Fischer Scientific	Cat#08-771-2
Falcon Cell strainer 40 µm	Fischer Scientific	Cat#08-771-1
BD FACSAria III	BD Biosciences	n/a

Lysis buffer (prepare the day of the experiment and keep it on ice)			
Reagent for lysis buffer	Final concentration	Amount	Storage conditions
Tris-HCl pH 7.4 (1 M)	10 mM	60 µL	Room temperature
NaCl (5 M)	10 mM	12 µL	Room temperature
$MgCl_2$ (1 M)	3 mM	18 µL	Room temperature
NonidetTM P40 (10%)	0.1%	60 µL	Room temperature (protected from light)
Nuclease-Free water	n/a	5.85 mL	
Total	**n/a**	**6 mL**	

Wash buffer (prepare the day of the experiment and keep it on ice)			
Reagent for wash buffer	Final concentration	Amount	Storage conditions
PBS, BSA 10%	2%	3 mL	4°C
PBS		12 mL	4°C
Total	**n/a**	**15 mL**	

Wash buffer with RNAse inhibitor (prepare the day of the experiment and keep it on ice)		
Reagent for wash buffer with RNAse inhibitor	Final concentration	Amount
Wash buffer with RNAse inhibitor	1X	3 mL
RNase inhibitor	0.2 U/μL	15 μL of stock 40 U/μL
Total	**n/a**	**3 mL**

PBS with RNAse inhibitor (prepare the day of the experiment and keep it on ice)		
Reagent for PBS with RNAse inhibitor	Final concentration	Amount
PBS with RNAse inhibitor	1X	1 mL
RNase inhibitor	0.2 U/μL	5 μL of stock 40 U/μL)
Total	**n/a**	**1 mL**

MATERIALS AND EQUIPMENT

FACS machine equipped with UV laser for nuclei purification.

10x Genomics Reagents

Step 1: Gem generation and barcoding

Chromium Controller & Next GEM Accessory Kit, 10x Genomics, 1000202

Chromium Next GEM Chip G Single Cell Kit, 16 rxns*, 10x Genomics, 1000127

Chromium Next GEM Single Cell 3′ GEM, Library & Gel Bead Kit v3.1, 16 rxns**, 10×Genomics, 1000121.

*available also in 48 rxns; ** available also in 4 rxns

Step 2: Post GEM-RT Cleanup & cDNA Amplification

Chromium Controller & Next GEM Accessory Kit, 1000202 (same kit as in step 1)

Chromium Next GEM Single Cell 3′ GEM, Library & Gel Bead Kit v3.1, 16 rxns, 1000121 (same kit as in step 1)

DynabeadsTM MyOneTM SILANE, 10x Genomics, 2000048 (store at 4°C)

SPRIselect Reagent Kit, Beckman Coulter, B23318

QIAGEN Buffer EB, QIAGEN, 19086

10% Tween 20, Bio-Rad, 1662404

100% EtOH + Nuclease-Free water for 80% EtOH

Step 3: 3′ Gene Expression Library Construction

Chromium Controller & Next GEM Accessory Kit, (same kit as in steps 1 and 2)

Chromium Next GEM Single Cell 3′ GEM, Library & Gel Bead Kit v3.1, 16 rxns*, 1000121 (same kit as in steps 1 and 2)

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Single Index Kit T Set A, 10x Genomics, 1000213

SPRIselect Reagent Kit, Beckman Coulter, B23318 (same as in step 2)

QIAGEN Buffer EB, QIAGEN, 19086 (same as in step 2)

100% EtOH + Nuclease-Free water from 80% EtOH

STEP-BY-STEP METHOD DETAILS

Nuclei purification

Timing: 1 h

During these steps, nuclei are extracted from mouse skeletal muscles. This protocol can be used with either fresh or frozen skeletal muscle. For freezing, remove all liquid (PBS or medium) and snap freeze the muscles in liquid nitrogen. Store the muscles in liquid nitrogen or a -150°C freezer. The day of the experiment, let the muscle thaw for 5 min on ice before adding ice-cold lysis buffer and proceeding with the nuclei purification.

1. Lysis of the tissue:
   a. Dissect the muscles of interest and place them in 1 mL of ice-cold PBS+RNase inhibitor in a 2 mL tube (Figure 1A).

   ⚠ CRITICAL: The amount of skeletal muscle used depends on the conditions (wild type resting muscle, regeneration, atrophy, etc.). For wild type, resting conditions the range of muscle quantity is 150–400 mg, equivalent to 3-6 adult tibialis anterior (TA) hindlimb muscles. It is strongly recommended to test different quantities of tissue before the experiment and check if the number of the sorted nuclei is sufficient.

   b. Discard the PBS and add 1 mL of ice-cold lysis buffer. Chop the tissue with a pair of clean dissection scissors for 2 min in the same 2 mL tube, keeping the tube on ice (Figure 1B). The resulting pieces of chopped muscle should be approximately 2–3 mm in diameter (Figure 1C).
   c. Transfer the chopped muscle into a 15 mL tube and add 4 mL of lysis buffer. Lyse for additional 3 min on ice with gentle shaking.
   d. Add 9 mL of wash buffer and transfer the preparation into the 15 mL Douncer. Place the Douncer on ice and while on ice, lower and raise the pestle gently 10 times, avoiding bubble formation (Figure 1D).

   ⚠ CRITICAL: The reciprocating motion of the pestle should be carried out quite effortlessly, without the need to apply strength; otherwise, this is an indication of excess muscle that will jeopardize nuclei integrity.

2. Filtration wash and staining:
   a. Filter the nuclei with a 70 μm cell strainer in a 50 mL conical tube. Before filtering, rinse the cell strainer with 1 mL of wash buffer without RNase inhibitor. Collect the flow through and re-filter using a 40 μm cell strainer in a clean 50 mL tube (pre-rinse the 40 μm cell strainer with 1 mL of wash buffer).
   b. Transfer the nuclei into a 15 mL tube. Centrifuge for 5 min at 500 *g*, 4°C (Figure 1E).

   ⚠ CRITICAL: The purification is good when the pellet is visible and clear in color (Figure 1E). If the pellet is less than 1/4 of what is shown in Figure 1E, the number of nuclei recovered after FACS may be insufficient. In this case, increase the amount of tissue used initially. If the

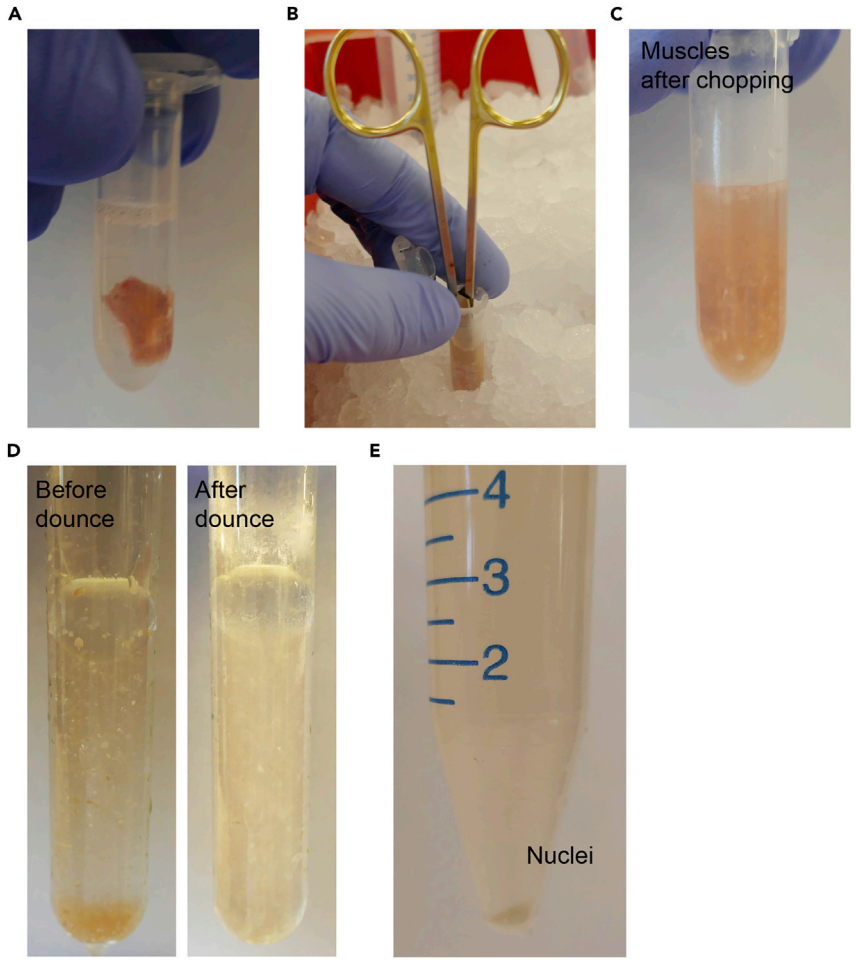


Figure 1. Nuclei extraction from adult skeletal muscle

(A) Amount of dissected skeletal muscle used for nuclei isolation.

(B) The muscles are chopped with a pair of scissors for 2 min on ice.

(C) Indicative picture of the muscles after chopping. The size of the muscle pieces should be around 1–2 mm.

(D) Muscle preparation before and after Douncer homogenization.

(E) Nuclei pellet after filtration and first centrifugation. A colorless pellet of the size shown in the picture is required.

pellet is too large and not white in color, reduce the initial amount of muscle. Excessive amounts of muscle may compromise the efficiency of nuclei extraction and increase the quantity of debris.

c. Gently discard the supernatant with an automatic pipetboy up to the 2 mL level and remove the remaining liquid with a P1000 pipette manually. Do not discard by inverting the tube.

d. Resuspend the pellet in 1 mL of wash buffer + RNase inhibitor by gently pipetting 5 times and transfer it in a clean DNA LoBind 1.5 mL tube to wash off the ambient RNA. Centrifuge 5 min at 500 *g*, 4°C.

e. Resuspend the pellet in 500 µL of wash buffer + RNase inhibitor by gently pipetting 5 times and add DAPI to a final concentration of 10 µg/mL.

f. Incubate for 5 min in the dark on ice with gentle shaking.

g. Add 1 mL wash buffer + RNase inhibitor, gently pipette and centrifuge 5 min at 500 *g*, 4°C. Resuspend the pellet by gently pipetting in 300 µL of wash buffer + RNase inhibitor.

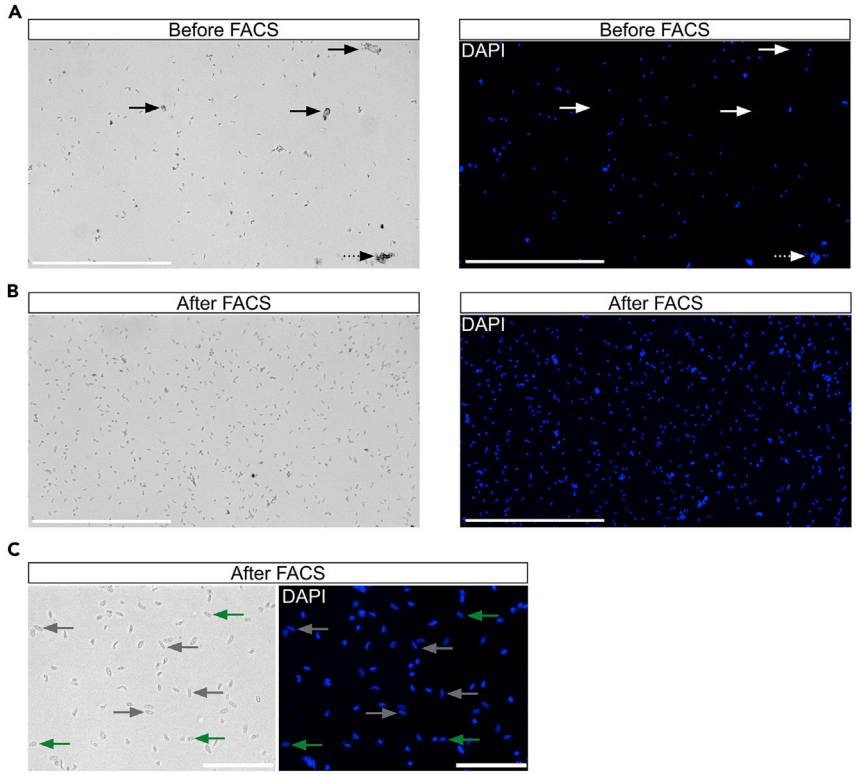


Figure 2. Nuclear purification by DAPI-based cytometry

(A) Nucleus preparations stained with DAPI after Douncer homogenization. Arrows point to debris (DAPI negative, solid arrows) and nuclei-debris aggregates (dotted arrow). Scale bar 400 μm.

(B) Nuclei preparations stained with DAPI after FACS. An almost complete elimination of debris is evident. Scale bar 400 μm.

(C) Nuclei from hindlimb and forelimb muscles of adult mice are sorted by FACS based on DAPI signal (≈ 50% DAPI+). Nuclei preparations stained with DAPI after FACS. Green arrows point to elongated nuclei, a characteristic feature of myonuclei, whereas gray arrows point at more rounded nuclei, probably deriving from other cell types. Scale bar 200 μm.

FACS sorting of nuclei

Timing: 30 min

During these steps, nuclei are purified by FACS based on DAPI staining.

3. FACS isolation of nuclei:
   a. Filter the nuclei in FACS tubes with a 35 μm nylon mesh-screen filter cap (rinse the filter with 50 μL of wash buffer + RNase inhibitor before and after filtering).
   b. Maintain a 1.5 mL collection tube at 4°C.
   c. FACS the nuclei based on the DAPI staining, with an 85 μm nozzle and a flow rate pressure of 45 psi. The nuclei should cluster separately from the debris (Figures 2A–2C). FACS sorting gates should be set to keep the nuclei population and exclude debris. Note: Unstained, DAPI-negative control nuclei could be used once for gating, although the signal-to-noise ratio is particularly high.
   d. Collect a minimum of 10 000 nuclei. Optimally, collect approximately 200 000 nuclei in 10 min.

Optional: At this stage, the nuclei preparation can be quality-controlled with brightfield or a fluorescent microscope to ensure that they are intact, of regular shape and that there is no

residual debris (Figures 2B and 2C). For brightfields microscope, mix 10 μl of nuclei with 10 μl of Trypan blue 0.4% and spread the nuclei on a glass slide, place a coverslip and visualize them. For fluorescent microscopy, directly spread the nuclei on a glass slide, place a coverslip and visualized them with a DAPI filter and in phase-contrast.

10x GEM generation, library preparation, and sequencing

Timing: 2 days

The RNA of each nucleus can be sequenced individually using either the Chromium Single Cell 3'Reagent Kits v3 or the Chromium Next GEM Single Cell 3'Reagent Kits v3.1. Nuclei are encapsulated into gel beads with individual barcodes using the Chromium Controller machine. Following encapsulation, cDNAs are manually generated and amplified and the library is prepared. The library is then sequenced and analyzed. These steps must be conducted by strictly following the manufacturer's protocol depending on the kit used. We will only present a summary of the first steps for the Chromium Next GEM Single Cell 3'Reagent Kits v3.1 thereafter.

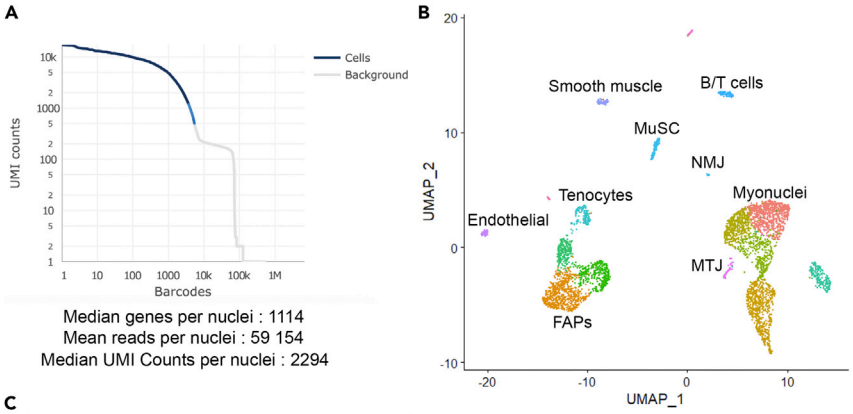
4. Encapsulation of nuclei:

Determine the volume of nuclei to be loaded into the 10X Chromium Controller. For this, divide the number of targeted nuclei times 1.6, by the concentration of nuclei after FACS (calculated during the test experiments). For example, if you target 5000 nuclei and have a concentration of 250 nuclei/μL after the FACS, the volume of nuclei to load into the machine is 5000*1.6/250 = 40 μL. As a general principle, the number of nuclei you load into the 10× Chromium Controller is twice the number you will target. For example, if you want to target 5000 nuclei, you should load 10 000. The maximum volume of sorted nuclei that can be loaded is 41.3 μL.

⚠ CRITICAL: If the concentration of nuclei is less than 200 nuclei/μl after the FACS, the preparation cannot be loaded directly into the 10X Genomics platform. In this case, FACS a minimum of 200 000 nuclei, centrifuge the nuclei at 500 g/10 min at 4°C, discard very gently the supernatant and resuspend the nuclei in a small volume of wash buffer + RNase inhibitor (for example 60 μl). Then precisely determine the concentration of nuclei with an automatic cell counter (like Countess 3 Automated Cell Counter from Thermo Fischer Scientific).

The rest of the protocol (GEM generation, RT, clean up, amplification, library construction and sequencing) must be carried out by strictly following the 10X Genomics protocol. In all conditions, the targeted number of sequencing reads per nucleus is 50 000.

5. Analysis:
   a. After sequencing, demultiplex the fastq files with the Cell Ranger mkfastq pipeline into library-specific FASTQ files. Cell Ranger is a set of analysis pipelines that process Chromium single-cell RNA-seq output to align reads, generate feature-barcode matrices and perform clustering and gene expression analysis. Cell Ranger is freely provided from 10x Genomics.
   b. The quantity of intronic reads is greater for the snRNA-seq compared to the scRNA-seq. It is therefore advisable to include these intronic reads in the analysis. For that, create a custom reference package including intronic and exonic reads, with cellranger mkgtf and cellranger mkref according to 10xGenomics instruction. Align the reads in the reference genome using STAR (Dobin et al., 2013)
   c. Once aligned, barcodes associated with these reads-cell identifiers and Unique Molecular Identifiers (UMIs), undergo filtering and correction. Reads associated with retained barcodes are quantified and used to build a transcript count table.



C

Population	Marker gene	Percentage of total nuclei
Body myonuclei	*Ttn*	61,8
FAPs	*Pdgfra*	23,4
Tenocyte	*Col11a1*	3,1
MuSC	*Pax7*	2,7
Endothelial	*Pecam*	2,0
Smooth muscular	*Myh11*	2,1
B/T Cells	*Ptprc*	2,3
Adipocytes	*Pparg*	0,7
MTJ myonuclei	*Ttn+Col22a1*	0,9
NMJ myonuclei	*Ttn+Ache*	0,3

Figure 3. Expected results after sequencing and analysis by Cell Ranger and Seurat

(A) Barcode rank plot showing the distribution of barcode counts and the barcodes associated with nuclei (from 10XGenomics). The curve must show a steep drop-off between the nuclei reads (left) and the background noise (right, degraded nuclei or empty partitions). If the nuclei preparation has too much contaminating ambient RNA, only one curve will be observed. The number of genes detected per nucleus must be between 700 and 2000. Y-axis: number of UMI counts mapped to each barcode; x-axis: number of barcodes.

(B) Uniform Manifold Approximation and Projection (UMAP) diagram from snRNA-seq from adult skeletal muscle. MuSC: skeletal muscle stem cells, FAPs: fibro-adipogenic progenitors, MTJ: myotendinous junction, NMJ: neuromuscular junction.

(C) Table of the different cell populations detected by snRNA-seq on skeletal muscle. The markers used to identify the different clusters are displayed in the middle and the percentage of nuclei for each population is displayed on the right.

d. It is advised to use SoupX software (Young and Behjati, 2020) to reduce the level of background of ambient RNA contamination. Use Seurat software (Stuart et al., 2019) to keep the nuclei with >200 and <2500 nFeature RNA and <5% mitochondrial genes.

Note: The parameters of filtering can be adjusted depending on the data quality and conditions.

e. Visualized, clustered and perform differential gene analysis using Seurat software.

EXPECTED OUTCOMES

The expected number of genes detected per nucleus is 750-1500 for nuclei isolated from adult skeletal muscle (Figure 3A).

The origin of the nuclei can be identified based on the expression of specific marker genes in wild type TA muscle (Figures 3B and 3C). The percentages of the cell population are shown in Figure 3. Around 65% of total nuclei are myonuclei expressing Titin (*Ttn*). Amongst these, slow-twitch fiber myonuclei express Myosin Heavy chain isoform *Myh7*, whereas nuclei of fast-twitch fibers express *Myh2*, *Myh1* and *Myh4*. Around 20% of total nuclei are fibro-adipogenic progenitor (FAP) nuclei and express Platelet Derived Growth Factor Receptor alpha (*Pdgfra*). The remaining nuclei are coming from endothelial cells (around 2%, expressing *Pecam1* (Platelet and Endothelial Cell

Adhesion Molecule 1)), muscle stem cell (around 2.5%, *Pax7*), adipocytes (around 1%, *Pparg* (Peroxisome Proliferator Activated Receptor gamma)), B/T cells (around 1%, *Ptprc* (Protein Tyrosine Phosphatase Receptor Type C), tenocytes (around 2.5%, expressing collagen *Col11a1*), smooth muscle cells (around 2%, *Myh11*), myotendinous junction myonuclei (around 1%, expressing collagen *Col22a1* and *Ttn*), and neuromuscular junction myonuclei (around 1%, expressing Acetylcholinesterase (*Ache*) and *Ttn*).

Compared to single-cell RNA-seq of skeletal muscles, single nuclei RNA-seq permit to capture the myonuclei. We observed that the percentage of FAPS is significantly higher in snRNA-seq datasets compared to scRNA-seq. On the contrary, the percentage of endothelial and immune cells (B and T cells) is systematically higher in the single-cell RNA-seq compared to the single-nuclei RNA-seq experiments.

We suggest that these discrepancies in cell population abundance could be due to differences in the efficiency of cell isolation during the enzymatic treatments and of nuclei extraction. Careful histological studies on biopsies combined with flow cytometry methods will help determine the actual population ratios.

LIMITATIONS

It has been shown that the nuclear transcriptome is representative of the whole-cell transcriptome (Gaidatzis et al., 2015). However, cytoplasmic RNAs, including Processing bodies (P-bodies) and other cytoplasmic ribonucleoprotein (RNP) residing transcripts, will not be detected by snRNA-seq. Since the quantity of total RNA in the nuclei is lower than in the cells (cytoplasmic + nuclear transcripts), snRNA-seq could impede the detection of low expressed genes. Another limitation is that for the isolation and sequencing of specific cell populations, genetic markers are required since no cell-surface markers can be used. On the other hand, nuclear markers could be used instead. Purification of nuclei by FACS is required to decrease the amount of ambient RNA encapsulated along with the nuclei. This introduces an additional step, however, FACS helps to decrease tissular debris, which is often a complication in single nucleus preparation.

If the number of nuclei encapsulated is too high, the number of nuclei doublets encapsulated in the same droplet may be too high. This can lead to the presence of false nuclei populations in Seurat analysis, often characterized by the presence of markers from different lineages. Moreover, snRNA-seq, like scRNA-seq, does not allow visualizing the spatial localization of the nuclei. It may be of interest to combine the results of snRNA-seq with immunohistochemistry, FISH experiments (for example RNAScope) or spatial transcriptomics.

Nuclear fluorescence signals can be lost during the extraction of the nuclei for certain genetic reporters. This is certainly the case for nuclear EGFP in the *Tg:Pax7-nGFP* mice that marks the muscle stem cells (Figures 4A–4D). The extent to which different fluorochromes are susceptible to the extraction conditions remains to be defined. Mild fixation of the cells preserved the GFP signal in the extracted nuclei (Figure 4C). However, in our hands, aldehyde fixation of cells is not compatible with 10x Genomics scRNA-seq (data not shown), and we have not assessed if fixed nuclei can be used for snRNA-seq.

TROUBLESHOOTING

Problem 1

After the FACS, the concentration of nuclei is less than 200 nuclei/μL and cannot be loaded directly into the 10X platform (step 3d).

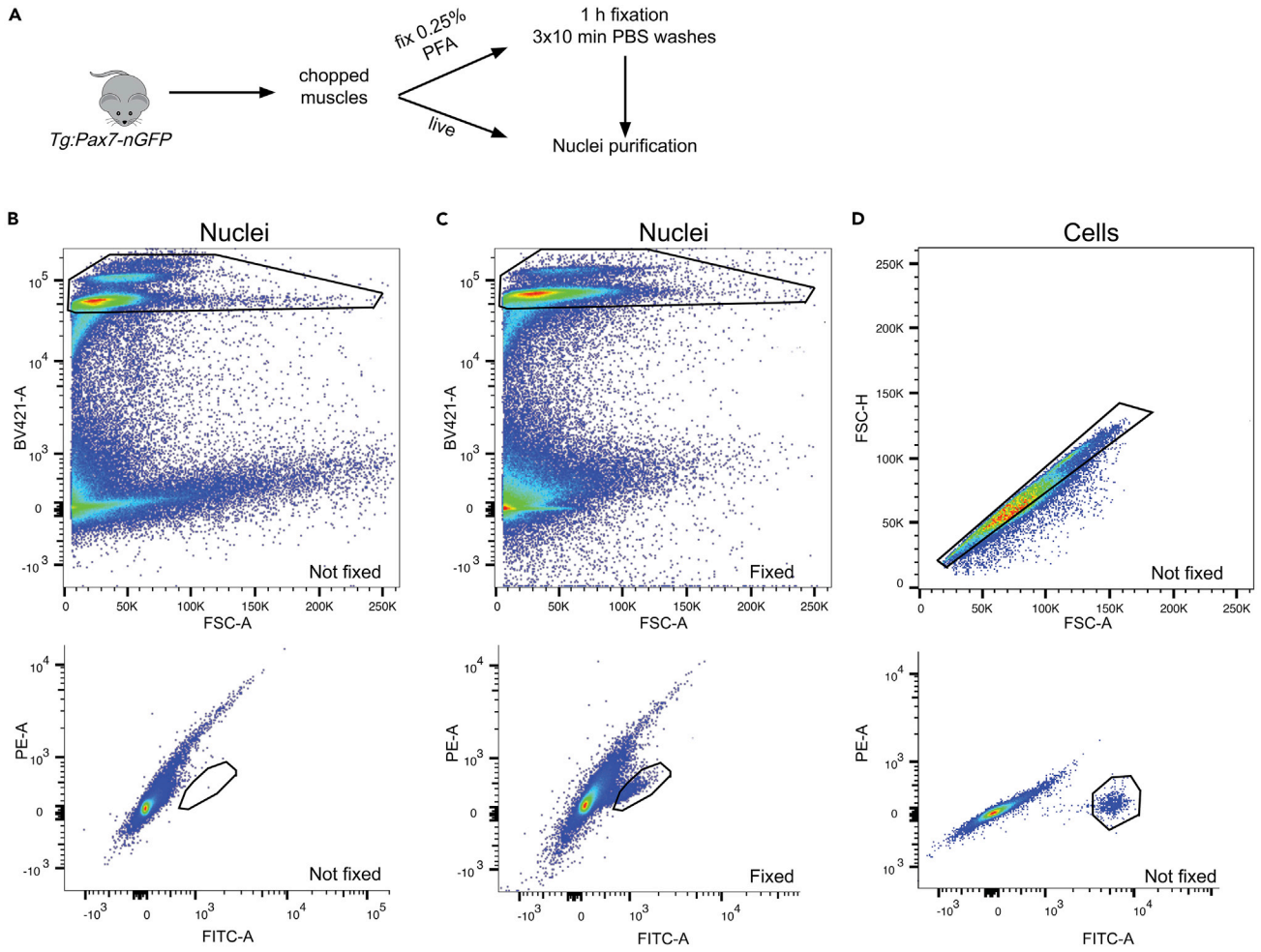


Figure 4. Nuclear GFP signal is lost during nuclei purification but can be preserved with mild formaldehyde fixation

(A) Forelimb and hindlimb muscles from 5.5-week-old *Tg:Pax7-nGFP* mice were manually dissected and chopped into small pieces and undergo nuclei purification with a Douncer homogenizer. For GFP preservation, samples are first fixed prior to homogenization in ice-cold 0.25% paraformaldehyde in PBS for 1 h at 4°C, then washed 3 × 10 min with PBS and are finally treated for FACS purification.

(B) Nuclei from not fixed samples sorted by FACS based on DAPI signal (65.1% DAPI+). Note that the nuclear GFP signal is undetectable.

(C) Nuclei from fixed samples sorted by FACS based on DAPI (55.7% DAPI+) and nuclear GFP signal (1.1% GFP+ of DAPI+).

(D) Nuclear GFP signal in single-cell preparations from *Tg:Pax7-nGFP* muscles allows sorting of GFP+ cells without the need for fixation (2.1% GFP+ of cell population).

Potential solution

FACS sort a minimum of 200 000 nuclei, centrifuge the nuclei at 500 g/10 min at 4°C with a swinging-bucket centrifuge, discard very gently the supernatant and resuspend the nuclei in a small volume of wash buffer + RNase inhibitor (for example 60 µL). Then precisely determine the concentration of nuclei with an automatic cell counter (like Countess 3 Automated Cell Counter from Thermo Fischer Scientific).

Important note when handling limited number of nuclei

If targeting a specific subpopulation of cells, it is likely that only a few nuclei, even less than 20 000, can be sorted in the limited time allowed to avoid artifacts. In this case, it is recommended to sort directly in an empty tube precoated with 2% BSA. After sorting, nuclei concentration must be reassessed by measuring the total volume of the sorted nuclei. Quality control and counting under the microscope with a hemocytometer must be carried out after sorting. To minimize the waste of nuclei

for counting, dilute 5 μL of nuclei preparation in 5 μL 2% BSA before loading on the hemocytometer. When handling less than 20 000 nuclei, centrifuging after sorting and re-diluting in a small volume will likely lead to loss of nuclei and is therefore highly discouraged.

Problem 2

The number of genes detected per nucleus is too low (step 5e).

Potential solution

Make sure that an RNase inhibitor was added in the buffers; visually verify nuclei's quality after FACS isolation; include the intronic sequence in the genome reference for reads alignment; verify that the number of reads sequenced per nucleus at least 50 000 reads.

RESOURCE AVAILABILITY

Lead contact

Dr Philippos Mourikis <philippos.mourikis@inserm.fr> is taking responsibility for the lead contact role.

Further information and requests for resources and reagents should be directed to the lead contact Dr Philippos Mourikis <philippos.mourikis@inserm.fr>.

Materials availability

No newly generated or specialized materials are required for the application of this protocol.

Data and code availability

The script used for the Seurat analysis is available at [https://github.com/matthieudossantos/Single-nuclei-RNAseq-and-single-nuclei-ATACseq-script-for-Seurat/blob/master/script_single_nuclei_RNAseq.r].

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AUTHOR CONTRIBUTIONS

M.D.S. tested and optimized the snRNA-seq protocol for murine skeletal muscle cells and wrote the manuscript. S.G. performed the experiments on GFP-labeled nuclei and wrote the manuscript. S.B. helped to optimize the protocol. M.D.S. and L.M. performed snRNA-seq experiments on intact and injured murine muscles. F.R., P. Maire, and P. Mourikis wrote and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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