Protocol to isolate and fix nuclei from flash frozen mouse gastrocnemius for IGVF V.2

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VERSION 2

NOV 08, 2023

OPEN ACCESS

Protocol Citation: Elisabeth Rebboah 2023. Protocol to isolate and fix nuclei from flash frozen mouse gastrocnemius for IGVF. **protocols.io** https://protocols.io/view/protocol-to-isolate-and-fix-nuclei-from-flash-froz-c4m2yu8eVersion created by Elisabeth Rebboah

Protocol status: Working
We use this protocol and it's working

Created: Nov 07, 2023

Last Modified: Nov 08, 2023

ABSTRACT

This protocol describes isolation of nuclei from 10 week old **left or right** mouse gastrocnemius muscle (tissue ID: 16) from 8 founder strains (B6J, AJ, 129S1J, NZOJ, WSBJ, NODJ, PWKJ, and CASTJ), preparation of a single nucleus suspension, and fixation for single nucleus RNA-seq using the Parse Biosciences protocol (Split-seq). We process 1 rep from each strain per day; e.g. female rep 1 across all 8 strains. For 8 samples, this protocol takes about 3.5 hours from start to finish.

The results is 1 aliquot of a fixed single-nucleus suspension for Parse Bio snRNA-seq ("Split-seq") from each of the 8 samples at >= 2,500 nuclei/ul stored at -80C.

The first part of the protocol describes tissue lysis and nuclei extraction using Miltenyi Biotec's gentleMACS Octo Dissociator with accessories. It also includes debris removal using Miltenyi Biotec's Debris Removal Solution and extra filtering steps specifically for working with skeletal muscle tissue. The second part describes nuclei fixation using Parse Biosciences Evercode Nuclei Fixation Kit with v2 reagents. Due to low nuclei recovery, we modify the original Parse Biosciences Evercode fixation protocol (attached) by using half volumes of all fixation reagents. We do not fix extra nuclei for other assays such as SHARE-seq, but save the whole left or right muscle.

ATTACHMENTS

SO+10122022_Evercode+Fixation+v2.0.2+User+Manual.pdf

GUIDELINES

1. Tilt tube and slowly add PBS during debris removal. Ideally, the cloudy debris is only in the band rather than the nuclei layer.
2. We recommend using a 5 mL pipette for aspirations and resuspensions > 1 mL.
3. Record everything in the IGVF spreadsheet, "Samples into experiment" tab.

MATERIALS

PROTOCOL integer ID:
90522

Keywords: Parse Biosciences, Fixation, Nuclei fixation, Gastrocnemius, Skeletal muscle, Muscle, Nuclei isolation, snRNA-seq, Evercode, Split-seq, Mouse, Mortazavi, IGVF, UCI

	Name	Manufacturer	Cat. #
	Nuclei Fixation Kit v2	Parse Biosciences	ECF2003
	Nuclei Extraction Buffer	Miltenyi Biotec	130-128-024
	RNase Inhibitor, murine	New England Biolabs	M0314L
	PBS	HyClone	SH30256.02
	Debris Removal Solution	Miltenyi Biotec	130-109-398
	7.5% BSA	Life Technologies	15260037
	gentleMACS C Tube	Miltenyi Biotec	130-093-237
	gentleMACS Octo Dissociator	Miltenyi Biotec	130-095-937
	MACS SmartStrainers (70 um)	Miltenyi Biotec	130-110-916
	MACS SmartStrainers (30 um)	Miltenyi Biotec	130-098-458
	pluriStrainer (20 um)	pluriSelect	43-50020-03
	NucBlue Fixed Cell ReadyProbes	Thermo Fisher	R37606
	Millicell Disposable Hemocytometer	Millipore	MDH-2N1-50PK
	Mr. Frosty	Sigma-Aldrich	635639

Reagents/equipment, manufacturer and catalog number

	Name	Reagent	Volume (for 8 samples)	Final Concentration
	Lysis buffer	Nuclei Extraction Buffer	35 ml	NA
		40 U/ul RNase inhibitor	175 ul	0.2 U/ul
	PBS	PBS	35 ml	NA
	HBSS	HBSS	20 ml	NA
	Debris Removal Solution (DRS)	Debris Removal Solution (Miltenyi)	8 ml	NA
	NB-BSA + RNase inhibitor	Nuclei Buffer (Parse Biosciences)	3.15 ml	NA
		7.5% BSA	350 ul	0.75%

	Name	Reagent	Volume (for 8 samples)	Final Concentration
		RNase inhibitor (Parse Biosciences)	44.1 ul	
	NB + RNase inhibitor	Nuclei Buffer (Parse Biosciences)	5 ml	NA
		RNase inhibitor (Parse Biosciences)	44.1 ul	
	RSB (x 2 aliquots!)	PBS	24.6 ml	NA
		7.5% BSA	333 ul	0.1%
		RNase inhibitor	125 ul	0.2 U/ul

Buffers

Setup

1 Label tubes.

2 Pre-chill centrifuge to **4C**.

3 Prepare 2 large ice buckets.

4 Prepare **35 ml lysis buffer** on ice in a 50 mL conical tube. Distribute **2 mL** into 8 gentleMACS C Tubes on ice. Add **175 ul RNase inhibitor** to the lysis buffer aliquot the day of the experiment.

5 Prepare **3.5 ml NB + BSA.** Add **44.1 ul RNase inhibitor** included in Parse Biosciences fixation kit the day of the experiment.

6 Prepare **50 mL RSB** on ice in 2 50 mL conical tubes. We keep a larger amount of PBS + 0.1% BSA at 4C, adding the RNase inhibitor the day of the experiment.

7 Prepare **5 ml nuclei buffer + RNase inhibitor** for final resuspension. Add **44.1 ul RNase inhibitor** to **5 ml nuclei buffer**.

8 Take an aliquot of PBS out of **4C** and keep on ice.

9 Take an aliquot of Debris Removal Solution out of **4C** and keep on ice.

10 Thaw components of **1 Parse Biosciences Nuclei Fixation kit** at room temperature, then place on ice.

11 Distribute **10 ul** NucBlue Fixed Cell ReadyProbes into 24 PCR strip tubes for cell counting. Need 8 tubes for counting after nuclei extraction, 8 tubes for counting after fixation, and another 8 tubes for filtered fixed nuclei.

Tissue lysis and nuclei extraction

12 Keep flash frozen tissue samples on dry ice.

13 Prepare 6 well plates on ice with ~**2 ml** of **HBSS** per well.

14 If necessary, drop both gastrocnemius tissues in a well, let them melt slightly, and separate them carefully using forceps.

15 Return one muscle to the sample tube and move the other to another labeled 1.5 ml tube. Flash-freeze both in liquid nitrogen.

16 Proceed with only one. Keep the other frozen in the same sample tube and return tubes to -80C box.

17 Drop left or right frozen tissue into a chilled gentleMACS C Tube with **2 mL lysis buffer.** Close tubes firmly and invert immediately, ensuring tissue is not stuck to the bottom or side. Keep tubes on ice and proceed immediately to dissociation.

18 Run the gentleMACS Program **4C_nuclei_1** on the Octo Dissociator (~**5 minutes**).

19 Remove tubes, ensuring tissue did not get stuck on the sides, and spin down in a 4C centrifuge for ~**10 seconds** to bring liquid to the bottom, then place tubes back on ice.

20 Filter nuclei suspension through **70 um** MACS SmartStrainer into a 5 mL tube. Fit a tube rack in ice for extra stability while filtering.

21 Wash **70 um** MACS SmartStrainer with **2 mL** additional lysis buffer. Add 2 mL to C tubes, cap, and swish to recover any nuclei stuck to the sides and cap of the C tubes, then wash the strainer.

22 Discard strainer and centrifuge the nuclei suspension at **4C**, **350g** for **5 minutes**.

23 Aspirate supernatant and resuspend nuclei pellet in **3.1 mL RSB**.

24 Filter nuclei suspension through **30 um** MACS SmartStrainer into a 15 mL tube.

25 Add **900 ul Debris Removal Solution** and mix by pipetting 10 times slowly up and down using a 5 mL pipette.

26 Overlay with **4 ml** PBS using a P1000 or 5 mL pipette (whichever you are more comfortable with).Tilt tube 45 degrees and slowly add the first mL. You can increase speed after the first mL of PBS is added.

27 Centrifuge at **4C**, **3000g** for **10 minutes** with full acceleration and no brake. Three phases are formed: top clear buffer layer, cloudy debris band, and clear layer containing nuclei. Pellet usually visible.

28 Aspirate the two top phases (buffer layer and all cloudy debris band) and discard. Aspirate the first phase, then the second phase. Stay above the third layer of nuclei to prevent loss. (See Fig. 1.)

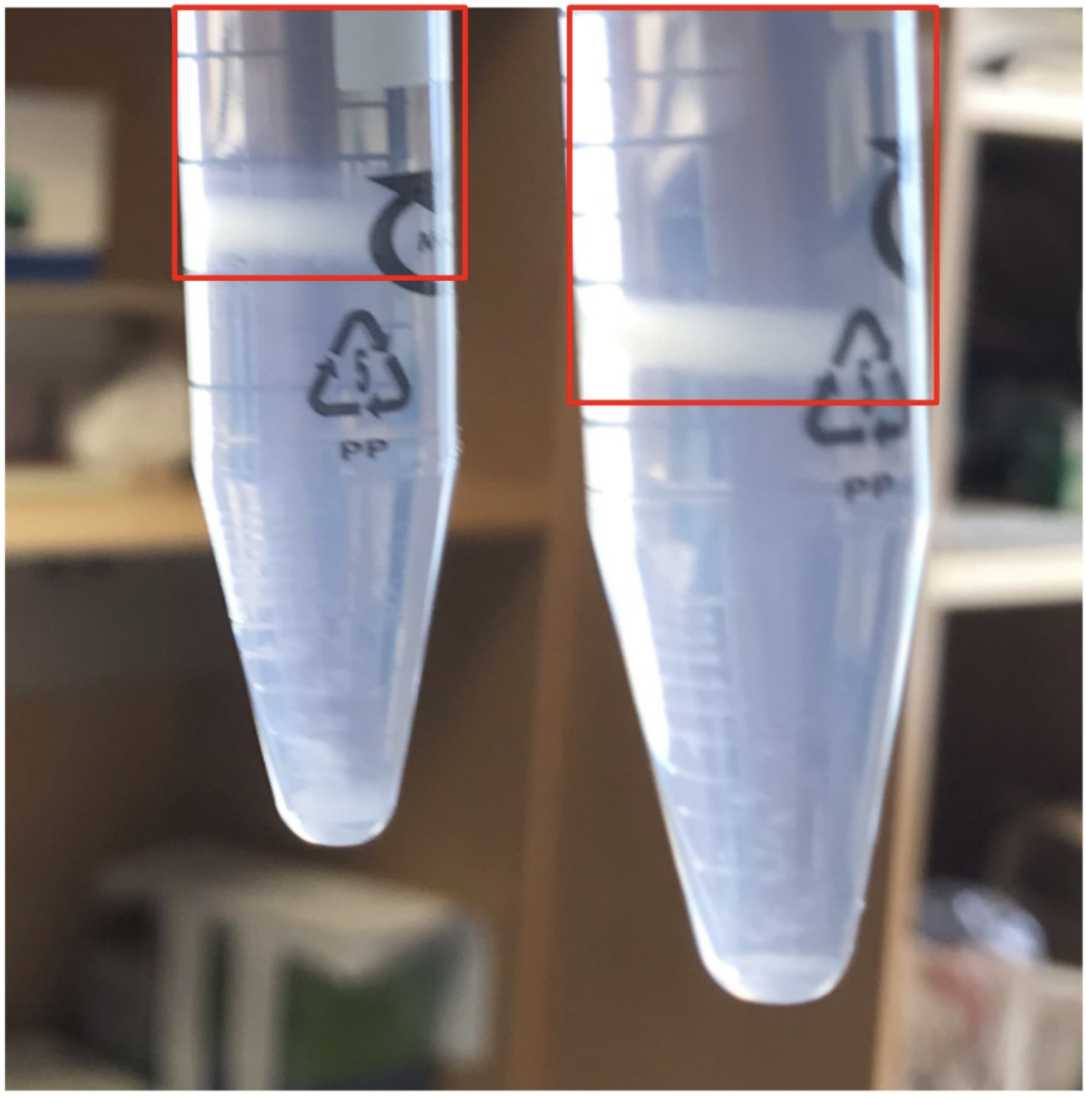

Fig. 1: Aspirate clear buffer layer and all of the cloudy debris layer outlined in red.

29 Fill with cold RSB to a final volume of **5 mL.**

30 Gently invert the tube three times. Do not vortex.

31 Centrifuge at **4C**, **1000g** for **10 minutes** with **full acceleration** and **full brake**.

32 Aspirate supernatant completely.

33 Resuspend cells carefully in **375 ul NB-BSA + RNase inhibitor** and filter through a 40 um strainer into a new 5 mL tube.

34 Count nuclei. Use **1:2** dilution factor, **10 ul + 10 ul dye.**

Nuclei fixation

35 Add **125 uL Nuclei Fixation Solution** to the filtered nuclei in 375 ul and mix 3 times. Do not over-mix.

36 Incubate nuclei for **10 minutes** on ice. Set 2 P200 pipettes to 40 ul and 125 ul.

37 Add **40 uL Nuclei Permeabilization Solution** and mix by pipetting 3 times with the P200 still set to 125 uL. Do not over-mix.

38 Incubate **3 minutes** with nuclei on ice.

39 Add **2 mL Nuclei Neutralization Solution** and invert the tube once to mix.

40 Centrifuge at **4C**, **750g** for **10 minutes**.

41 Aspirate and discard supernatant.

42 Resuspend the samples in **500 uL Nuclei Buffer with RNase inhibitor** without BSA. Check concentration with a hemocytometer under the microscope. Use **1:2** dilution factor, **10 ul + 10 ul dye**.

43 Filter nuclei through a **20 um filter** in 1, 2, 3, or 4 rounds depending on the amount of debris. Place filter in labeled 1.5 ml tube and dispense nuclei in 500 ul on top. Centrifuge at 4C, 200g for 1 minute to pull the solution through the filter. Repeat step if necessary, using a new filter for each round. Our reasoning is to prevent clogging by filtering in multiple rounds, but yield decreases by 90% before and after fixation, mostly due to the filtration at this step.

44 Take a **10 ul** aliquot to dilute **1:2** with prepared **10 ul dye** to manually count with a disposable hemacytometer and record numbers.

45 Count nuclei. Use **1:2** dilution factor, **10 ul + 10 ul dye.**

46 Re-concentrate: spin nuclei **750g** for **5 minutes** and carefully take off supernatant until **50 ul** are remaining. Resuspend (hopefully visible) pellet in the remaining **50 ul.**

47 Add Nuclei DMSO: **1 ul** into 50 ul samples and gently flick tubes to mix. One minute later, add another **1 ul** and flick to mix, then after another minute add a final **1 ul** for a total volume of **3 ul**. Mix by gently pipetting 5x with a P200 set to 25 ul.

48 Place tubes in a Mr. Frosty for storage at -**80C**. The next day, move tubes to boxes in -80C racks.