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

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AUG 25, 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-cy7jxzkn

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

Created: Aug 25, 2023

Last Modified: Aug 25, 2023

PROTOCOL integer ID: 86987

ABSTRACT

This protocol describes isolation of nuclei from 10 week old **left or right** mouse gastrocnemius muscle 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. We process 1 rep from each strain per day; e.g. female rep 1 across all 8 strains. The main products we use are Parse Biosciences Nuclei Fixation Kit (v2) and Miltenyi Biotec's gentleMACS Octo Dissociator with accessories. This protocol takes about 3.5 hours from start to finish.

The results are 1 aliquot of fixed single-nucleus suspensions for Parse per each of the 8 samples at >= 2,500 nuclei/ul.

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

	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

	Name	Manufacturer	Cat. #
	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 Sigma	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%
		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 Pre-chill centrifuge to 4C.

2 Prepare 2 large ice buckets.

3 Prepare lysis buffer on ice in a 50 mL conical tube. Distribute 2 mL into 8 gentleMACS C Tubes on ice.

4 Prepare NB + BSA + RNAse inhibitor in a 5 mL tube.

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

6 Prepare NB + RNase inhibitor in a 5 mL tube for final resuspension.

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

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

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

10 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

11 Keep flash frozen tissue samples on dry ice.

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

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

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

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

16 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.

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

18 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.

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

20 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.

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

22 Aspirate supernatant and resuspend nuclei pellet in 3.1 mL RSB.

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

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

25 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.

26 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.

27 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.)

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

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

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

31 Aspirate supernatant completely.

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

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

Nuclei fixation

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

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

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

37 Incubate 3 minutes with nuclei on ice.

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

39 Centrifuge at 4C, 750g for 10 minutes.

40 Aspirate and discard supernatant.

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

42 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.

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

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

45 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.

46 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.

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