Protocol to isolate and fix nuclei from flash frozen mouse tibialis anterior for IGVF V.1

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

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ABSTRACT

This protocol describes isolation of nuclei from 10 week old mouse tibialis anterior 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 2 aliquots of fixed single-nucleus suspensions for Parse per each of the 8 samples.

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
	gentleMACS C Tube	Miltenyi Biotec	130-093-237

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	Name	Manufacturer	Cat. #
	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
	NucBlue Fixed Cell ReadyProbes	Thermo Fisher	R37606
	Hemacytometer	Fisher Scientific	02-671-51B
	Mr. Frosty	Sigma-Aldrich	635639

	Name	Reagent	Volume (for 8 samples)	Final Concentration
	Lysis buffer	Nuclei Extraction Buffer	40 ml	NA
		40 U/ul RNase inhibitor	200 ul	0.2 U/ul
	PBS	PBS	25 ml	NA
	Debris Removal Solution (DRS)	Debris Removal Solution (Miltenyi)	8 ml	NA
	NB + BSA	Nuclei Buffer (Parse Biosciences)	3.6 ml	NA
		7.5% BSA	400 ul	0.75%
		RNase inhibitor (Parse Biosciences)	20 ul	
	RSB	PBS	24.6 ml	NA
		7.5% BSA	333 ul	0.1%
		RNase inhibitor	125 ul	0.2 U/ul

Buffer

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 2.5 mL into 8 gentleMACS C Tubes on ice.

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

5 Prepare 25 mL RSB on ice in a 50 mL conical tube (need ~3 mL per sample). We keep a larger amount of PBS + 0.1% BSA at 4C, adding the RNase inhibitor the day of the experiment.

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

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

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

9 Distribute 10 ul NucBlue Fixed Cell ReadyProbes into 16 PCR strip tubes for cell counting. Need 8

tubes for counting after nuclei extraction, and another 8 tubes for final fixed nuclei.

Tissue lysis and nuclei extraction

10 Keep flash frozen tissue samples on dry ice until lysis.

11 Drop whole frozen tissue into a chilled gentleMACS C Tube with 2.5 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.

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

13 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.

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

15 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.

16 Discard strainer and centrifuge the 4.5 mL nuclei suspension at 4C, 350g for 5 minutes.

17 Resuspend nuclei pellet in 3.1 mL PBS.

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

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

20 Overlay with 4 ml PBS using a P1000 or 5 mL pipette. Tilt tube 45 degrees and slowly add the first mL. You can increase speed after the first mL of PBS is added.

21 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 not visible.

22 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.

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

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

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

26 Aspirate supernatant completely.

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

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

Nuclei Fixation

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

30 Incubate nuclei for 10 minutes on ice. Set 2 P200 pipettes to 40 ul and 250 ul.

31 Add 40 uL Nuclei Permeabilization Solution and mix by pipetting 3 times with the P200 still set to 250 uL. Do not over-mix.

32 Incubate 3 minutes with nuclei on ice.

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

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

35 Aspirate and discard supernatant.

36 Resuspend the samples in 100 uL Nuclei Buffer without BSA. If there is excessive debris and we can afford to add more volume (>= 100 uL), then filter through a 40 um strainer into a new 1.5 mL tube. With less than 200 uL, too much of the sample gets lost in the filter.

37 Take a 10 ul aliquot to dilute 1:2 with prepared 10 ul DAPI to manually count with a hemacytometer and record numbers.

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

39 Add Nuclei DMSO: 1.7 ul into 100 ul samples and gently flick tubes to mix. One minute later, add another 1.7 ul and flick to mix, then after another minute add a final 1.7 ul for a total volume of ~5 ul. Mix by gently pipetting 5x.

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