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

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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 kidneys 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 Parse Biosciences. 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

- We recommend using a 5 ml pipette for aspirations and resuspensions > 1 ml.
- 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
	7.5% BSA	Life Technologies	15260037
	gentleMACS C Tube	Miltenyi Biotec	130-093-237
	gentleMACS Octo Dissociator	Miltenyi Biotec	130-095-937

	Name	Manufacturer	Cat #
	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

Reagents/equipment, manufacturer and catalog number

	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
	NB + BSA + RNase inhibitor(make 2 aliquots)	Nuclei Buffer (Parse Biosciences)	3.15 ml	NA
		7.5% BSA	350 ul	0.75%
		RNase inhibitor (Parse Biosciences)	RNase inhibitor (Parse Biosciences)	44.1 ul
	RSB	PBS	42 ml	
		7.5% BSA	560 ul	0.1%
		RNase inhibitor	210 ul	0.2 U/ul
	SHARE-RSB	1 M HEPES pH 7.3	150 ul	10 mM
		5 M NaCl	30 ul	10 mM
		1 M MgCl2	45 ul	3 mM
		10% Tween-20	150 ul	0.1%
		H2O	14.625 ml	
		7.5% BSA	80.26 ul	0.04%
		5% digitonin	30 ul	0.01%
		Enzymatics RI	37.5 ul	0.1 U/ul
		SUPERase RI	18.75 ul	0.025 U/ul

	Name	reagent	Volume for 8 samples	Final concentration
		Yeast tRNA	150 ul	100 ug/ml

Buffers

Setup

1 Label tubes.

2 Pre-chill centrifuge to 4C.

3 Prepare ice buckets.

4 Prepare lysis buffer in a 50 ml conical tube on ice. Distribute 2.5 ml into 8 gentleMACS C Tubes on ice. Add RNase inhibitor to the lysis buffer aliquot the day of the experiment.

5 Prepare RSB in a 50 ml conical tube on ice. Add NEB RNase inhibitor the day of the experiment.

6 Prepare NB + BSA + RNase inhibitor. Add RNase inhibitor included in Parse Biosciences fixation kit the day of the experiment.

7 Prepare 2.5 ml Nuclei Buffer + RNase inhibitor for final resuspension. Add 31.5 ul Parse RNase inhibitor to 2.5 ml nuclei buffer.

8 Thaw components of 2 Parse Biosciences Nuclei Fixation v2 kits at room temperature, then place on ice.

9 Distribute 20 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. There should be 2 kidneys.

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 Discard supernatant and resuspend nuclei pellet in 3 ml RSB. Filter nuclei suspension through 30 um MACS SmartStrainer into a 5 ml tube.

18 Take 200 ul and add to a 5 ml tube that contains another 1.8 ml RSB for a 1:10 dilution (same as male gonads and similar to liver protocols).

19 Count nuclei. Use 1:11 dilution factor, 2 ul + 20 ul dye.

20 Centrifuge 4 million nuclei at 4C, 500g for 5 minutes and remove supernatant.

21 Resuspend pellet in 750 ul NB-BSA + RNase inhibitor.

Parse nuclei fixation

22 Filter the nuclei suspension (750 ul) NB-BSA + RNase inhibitor through a 40 um strainer (provided in Parse Biosciences kit) into a new 5 ml tube.

23 Add 250 uL Nuclei Fixation Solution and mix 3 times. Do not over-mix.

24 Incubate nuclei for 10 minutes on ice. Set 1 P200 pipette to 80 ul and keep the P1000 at 250 ul.

25 Add 80 uL Nuclei Permeabilization Solution and mix by pipetting 3 times with the P1000 still set to 250 uL. Do not over-mix.

26 Incubate 3 minutes with nuclei on ice.

27 Add 4 ml Nuclei Neutralization Solution and invert the tube once to mix.

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

29 Aspirate and discard supernatant.

30 Resuspend the samples in 300 ul Nuclei Buffer with RNase inhibitor without BSA and filter through a 40 um filter into a labeled 1.5 ml tube.

31 Count nuclei. Use a 1:11 dilution factor, e.g. 2 ul + 20 ul dye.

32 Add Nuclei DMSO: For 300 ul samples: add 5 ul and gently flick tubes to mix. One minute later, add another 5 ul and flick to mix, then after another minute add a final 5 ul for a total volume of 15 ul. Mix by gently pipetting 5x with a P200 set to 150 ul.

33 Split nuclei suspension into 2 aliquots of equal volume in labeled 1.5 ml tubes.

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

35 Move leftover nuclei suspension to labeled 2 ml tubes and spin at 4C, 750g for 5 minutes. Remove supernatant and flash-freeze nuclei in liquid nitrogen as dry pellets. Store at -80C.

SHARE-seq nuclei fixation

36 Set aside 1 million nuclei for each of the 8 samples in RSB and spin down at 4C, 750g for 5 minutes.

37 Remove supernatant and resuspend nuclei pellet in 1 ml room temperature SHARE-RSB. Transfer tube to a room temperature rack.

38 At RT, add 13.34 ul of methanol-free formaldehyde (16% stock solution). Final concentration for nuclei: 0.2%. Close tube and nutate cells at RT for 5 minutes.

39 To quench fixation, per reaction, add 56.1 ul fresh 2.5M Glycine (0.94g per 5 ml stock), 50 ul of 1M Tris pH 8.0, 13.3ul of 7.5% BSA, and mix using a pipette. Incubate on ice for 10 minutes.

40 Spin 750g, 4C, 5 minutes. Gently remove supernatant.

41 Add 200 ul of SHARE-RSB and gently resuspend pellet. Store on ice until all samples are

completed.

42 Pool 200 ul of resuspended nuclei from all 8 founders into 1 labeled 2 ml tube.

43 Spin 1,000g, 4C, 10 minutes. Gently remove supernatant. Remove all fluid and freeze at -80C as a dry pellet.

Storage of leftover nuclei

44 Move remaining nuclei in RSB in 5 ml tubes on ice to new labeled 2 ml tubes.

45 Spin 750g, 4C, 5 minutes.

46 Remove all supernatant and flash-freeze nuclei as a dry pellet in liquid nitrogen. Store at -80C.