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We use this protocol and it's working

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

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ABSTRACT

This protocol describes isolation of nuclei from 10 week old whole mouse livers 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 and SHARE-seq using part of the Broad Institute 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, and 1 fixed nuclei pellet pooled across all 8 strains for SHARE-seq.

GUIDELINES

- We recommend using a 5 ml pipette for aspirations and resuspensions > 1 ml.
- We cut the frozen livers ahead of time on dry ice to make lysis easier.
- Tips to get frozen chopped tissues out of tubes: **Use forceps** or a 1 ml tip to break up frozen tissue pieces so they can fit on the sides of the C tube. Tap tubes firmly upside-down on bench. Flick/shake open tube over the C tube with lysis buffer. Flick/shake C tube with liver pieces so that they are at least touching the lysis buffer.
- Record everything in the IGVF spreadsheet, "Samples into experiment" tab.
- When possible, after nuclei isolation and during the first round of counting, 2 technicians should continue on with Parse fixation after establishing the volume needed for roughly 4 million nuclei per sample (4 samples processed per technician). The remaining technician should determine the exact volume needed for 1 million cells and proceed with SHARE-seq fixation. Parallel fixation saves about an hour of time.

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
	1 M HEPES pH 7.3	Sigma	H0887-100ml
	NaCl	Fisher	BP358-1
	MgCl2	Fisher	AA12315A7
	Tween-20	Fisher	BP337-500
	5% digitonin	Promega	G944A
	Enzymatics RI	Enzymatics	Y9240L
	SUPERase RI	Invitrogen	AM2696
	Yeast tRNA	Invitrogen	AM7119
	Glycine	Fisher	BP381-500
	1M Tris pH 8.0	Thermo	AM9855G
	Formaldehyde (methanol-free)	EMS	15710
	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
	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
	1% BSA-DEPC	BSA	1 g	1%
		DEPC water	100 ml	
	Lysis buffer	Nuclei Extraction Buffer	50 ml	
		40 U/ul RNase inhibitor	250 ul	0.2 U/ul
	NB + BSA	Nuclei Buffer (Parse Biosciences)	3.5 ml	
		7.5% BSA	350 ul	0.75%
		RNase inhibitor (Parse Biosciences)	44.1 ul	
	RSB (Make 2 aliquots of 35 ml = 70 ml for 8 samples)	PBS	35 ml	
		7.5% BSA	467 ul	0.1%
		RNase inhibitor	175 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
		Yeast tRNA	150 ul	100 ug/ml

Buffers

Setup

1 Coat SHARE-seq nuclei prep tubes with BSA. Fill 8 1.5 ml tubes with 1.5 ml 1% BSA in H2O and incubate for 30 minutes. After incubation, aspirate BSA solution and dry for 30 minutes. Store at 4C.

2 Label tubes.

3 Pre-chill centrifuge to 4C.

4 Prepare ice buckets.

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

6 Prepare RSB in 2 50 ml conical tubes on ice, 35 ml per tube. Add RNase inhibitor the day of the experiment.

7 Prepare 2 x 3.5 ml nuclei buffer + BSA + RNase inhibitor (NB + BSA).

8 Prepare 2.5 ml nuclei buffer + RNase inhibitor for final resuspension. Add 31.5 ul RNase inhibitor to 2.5 ml nuclei buffer.

9 Prepare 15 ml SHARE-RSB in a 50 ml conical tube at room temperature. 30 ul digitonin, 37.5 ul

Enzymatics RI, 18.75 ul SUPERase RI, and 150 ul yeast tRNA.

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

11 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 sectioning

12 Keep flash frozen tissue samples on dry ice.

13 Tilt frozen tissues into a plastic petri dish on dry ice.

14 Using a clean razor blade, roughly chop tissue into pieces ~500 mg. Not necessary to chop tissues < 500 mg.

15 Using clean forceps, move chopped, frozen tissue back to the original 2 ml tube. Tissue should never be thawed during this process.

Tissue lysis and nuclei extraction

16 Keep chopped flash frozen tissue samples on dry ice until lysis.

17 Drop whole frozen tissue into a chilled gentleMACS C Tube with 3.5 ml lysis buffer. For NZO

mice, use 4 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 15 ml tube. Fit a tube rack in ice for extra stability while filtering.

21 Wash 70 um MACS SmartStrainer with 2.5 ml additional lysis buffer. Add 2.5 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 4 ml nuclei suspension at 4C, 350g for 5 minutes.

23 Resuspend nuclei pellet in 3.5 ml RSB.

24 Filter nuclei suspension through 30 um MACS SmartStrainer into a 5 ml tube.

25 Dilute some nuclei 1:10 by adding 500 ul nuclei to 4.5 ml RSB. This should help the concentration reach around 4 million per ml.

26 Count 1:10 diluted nuclei. Use 1:5 dilution factor, e.g. 5 ul + 20 ul dye. Final dilution factor = 50.

Parse nuclei fixation

27 Set aside 4 million nuclei in RSB in a new 5 ml tube and spin down at 4C, 550g for 5 minutes.

28 Remove supernatant and and resuspend nuclei in 750 ul NB + BSA + RNase inhibitor through a 40 um strainer (provided in Parse Biosciences kit) into a new 5 ml tube.

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

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

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

32 Incubate 3 minutes with nuclei on ice.

33 Add 4 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 300 ul Nuclei Buffer with RNase inhibitor without BSA and move through a 40 um filter into a labeled 1.5 ml tube.

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

38 Add Nuclei DMSO: 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 125 ul.

39 Split nuclei suspension into 2 labeled tubes, 150 ul per tube.

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

41 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

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

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

44 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.

45 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.

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

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

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

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

50 Move remaining nuclei in RSB on ice to labeled 2 ml tubes.

51 Spin 750g, 4C, 5 minutes.

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