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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 male mouse gonads for IGVF

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ABSTRACT

This protocol describes isolation of nuclei from left **and** right 10 week old mouse testes and epididymis (pooled tissue ID: 25) from 8 founder strains (B6J, AJ, 129S1J, NZOJ, WSBJ, NODJ, PWKJ, and CASTJ), preparation of a single nucleus suspension, and fixation for 1. single nucleus RNA-seq using the Parse Biosciences protocol (Split-seq) and 2. single nucleus RNA-seq + ATAC-seq using the SHARE-seq protocol. We process 1 rep from each strain per day; e.g. male rep 1 across all 8 strains. For 8 samples, 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 at >= 2,500 nuclei/ul, and 1 fixed nuclei pellet pooled across all 8 strains for SHARE-seq, all stored at -80C.

The first part of the protocol describes tissue lysis and nuclei extraction using Miltenyi Biotec's gentleMACS Octo Dissociator with accessories. When nuclei are extracted and counted, we determine whether we have enough to fix for Split-seq and SHARE-seq and set aside 4 million and 1 million, respectively. Ideally, the second and third parts of this protocol are performed in parallel by at least two technicians to save time. The second part describes nuclei fixation using Parse Biosciences Evercode Nuclei Fixation Kit with v2 reagents (see attachment for original version). The third part describes nuclei fixation using a modified version of the SHARE-seq fixation protocol (see attachment for original version). Any remaining nuclei are flash-frozen as a dry pellet and stored at -80C.

ATTACHMENTS

2022_07_15_GRO_nuclei_prep_combo.docx

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

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

Keywords: Fixation, Nuclei fixation, Split-seq, SHARE-seq, Evercode, snRNA-seq, Parse Biosciences, Nuclei isolation, UCI, Mortazavi, IGVF, Mouse, Gonads, Testes, Epididymis, Testes/epididymis, Male gonads

	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
	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)	44.1 ul	
	RSB	PBS	24.6 ml	
		7.5% BSA	333 ul	0.1%
		RNase inhibitor	125 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 **40 ml lysis buffer** in a 50 ml conical tube on ice. Distribute **2.5 ml** into 8 gentleMACS C Tubes on ice. Add **200 ul RNase inhibitor** to the lysis buffer aliquot the day of the experiment.

6 Prepare **25 ml RSB** in a 50 ml conical tube on ice. Add **125 ul RNase inhibitor** the day of the experiment.

7 Prepare **2 aliquots of 3.5 ml NB + BSA.** Add **44.1 ul RNase inhibitor** included in Parse Biosciences fixation kit the day of the experiment to each aliquot.

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. To SHARE-RSB, add **30 ul digitonin**, **37.5 ul Enzymatics RI**, **18.75 ul SUPERase RI,** and **150 ul yeast tRNA** fresh the day of the experiment**.**

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 lysis and nuclei extraction

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

13 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 4 pieces: left and right testes and left and right epididymis.

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

15 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.

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

17 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.

18 Discard strainer and centrifuge the 4.5 ml nuclei suspension at **4C**, **350g** for **5 minutes**.

19 Discard supernatant and resuspend nuclei pellet in **3 ml RSB**.

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

21 Dilute some nuclei **1:20** by adding **100 ul nuclei** to **1.9 ml RSB** in a new 5 mL tube. This should help the concentration reach around 4 million per ml.

22 Count 1:20 diluted nuclei. Use **1:5** dilution factor, **2 ul + 20 ul dye.** Final dilution factor = 1:100.

Parse nuclei fixation

23 Set aside **4 million nuclei in RSB** in a new 5 ml tube and spin down at **4C**, **350g** for **5 minutes**.

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

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

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

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

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

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

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

31 Aspirate and discard supernatant.

32 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.

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

34 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.

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

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

SHARE-seq nuclei fixation

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

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

39 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**.

40 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**.

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

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

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

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

Storage of leftover nuclei

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

46 Spin **750g**, **4C**, **5 minutes**.

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