Protocol to isolate and fix nuclei from cryopreserved left cortex mouse bridge samples 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 left cerebral cortex from B6J and CASTJ, preparation of a single nucleus suspension, and fixation for single nucleus RNA-seq using Parse Biosciences. We process 2 reps from each strain per day; e.g. both male and female reps across both 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 main difference from our other nuclei prep protocols is storage of the tissue: for these mouse bridge samples, tissues were stored in 1 mL Bambanker media in cryotubes.

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

The first part of the protocol describes tissue lysis and nuclei extraction using Miltenyi Biotec's gentleMACS Octo Dissociator with accessories. The second part describes nuclei fixation using Parse Biosciences Evercode Nuclei Fixation Kit with v2 reagents.

ATTACHMENTS

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

GUIDELINES

1. We recommend using a 5 ml pipette for aspirations and resuspensions > 1 ml.
2. Record everything in the IGVF spreadsheet, "Samples into experiment" tab.

MATERIALS

	Name	Manufacturer	Cat. #
	Nuclei Fixation Kit v2	Parse Biosciences	ECF2003

Keywords: Fixation, Nuclei fixation, Split-seq, Evercode, snRNA-seq, Parse Biosciences, Nuclei isolation, UCI, Mortazavi, IGVF, Mouse, Mouse brain, Bridge samples, IGVF bridge, Left cortex, Cortex

	Name	Manufacturer	Cat. #
	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
	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	35 ml	NA
		40 U/ul RNase inhibitor	175 ul	0.2 U/ul
	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	
	RSB	PBS	24.6 ml	
		7.5% BSA	333 ul	0.1%
		RNase inhibitor	125 ul	0.2 U/ul

Buffers

Setup

1 Label tubes.

2 Pre-chill centrifuge to **4C**.

3 Prepare ice buckets.

4 Prepare **35 ml lysis buffer** on ice in a 50 mL conical tube. Distribute **2 mL** into 8 gentleMACS C Tubes on ice. Add **175 ul RNase inhibitor** to the lysis buffer aliquot the day of the experiment.

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

6 Prepare **3.5 ml NB + BSA.** Add **44.1 ul RNase inhibitor** included in Parse Biosciences fixation kit the day of the experiment.

7 Prepare **1.5 ml nuclei buffer + RNase inhibitor** for final resuspension. Add **18.6 ul RNase inhibitor** to **1.5 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 Thaw tissues in Bambanker media on ice until the tissue can be extracted.

11 Transfer the tissue to a chilled gentleMACS C Tube with **2 ml lysis buffer** using forceps. 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 ml nuclei suspension at **4C**, **350g** for **5 minutes**.

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

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

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

Parse Nuclei Fixation

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

21 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.

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

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

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

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

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

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

28 Aspirate and discard supernatant.

29 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.

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

31 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 150 ul.

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

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

Storage of leftover nuclei

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

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

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