Feb 01, 2025

Protocol to isolate and fix nuclei from flash frozen mouse brain for IGVF SHARE-seq

DOI
https://dx.doi.org/10.17504/protocols.io.5jyl8dq29g2w/v1

Elisabeth Rebboah[1]

[1]University of California, Irvine

IGVF

Elisabeth Rebboah
University of California, Irvine

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External link: https://static.miltenyibiotec.com/asset/150655405641/document_fks46sfs0p7c79hgi64bqfa33d?content-disposition=inline

Protocol Citation: Elisabeth Rebboah 2025. Protocol to isolate and fix nuclei from flash frozen mouse brain for IGVF SHARE-seq . **protocols.io** https://dx.doi.org/10.17504/protocols.io.5jyl8dq29g2w/v1

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

Created: January 31, 2025

Last Modified: February 01, 2025

Protocol Integer ID: 119404

Keywords: fixation, nuclei fixation, SHARE-seq, mouse brain, nuclei isolation, Mouse, nuclei from flash frozen mouse brain, flash frozen mouse brain, protocol with mouse brain cortex, isolation of nuclei, single nucleus suspension, mouse brain cortex, selection for nuclei, nuclei, castj mice, mice, brain tissue, igvf share, mouse

Abstract

This protocol describes isolation of nuclei from brain tissues of 10-week-old B6J and CASTJ mice, preparation of a single nucleus suspension, selection for nuclei using Miltenyi Biotec's Anti-Nucleus MicroBeads, and fixation for SHARE-seq using part of the Broad Institute protocol. We processed **8 samples at once** (2 males and 2 females per strain) with **two technicians** in parallel. This protocol takes about **3 hours** from start to finish. We have successfully tested this protocol with mouse brain cortex (either left or right side) and diencephalon (thalamus+hypothalamus)+pituitary.

Attachments

DOCX

Protocol to isolate ...

15KB

Guidelines

1. Buffers can be prepared ahead of time and kept in 4°C. Add RNase inhibitors fresh the day of the experiment.
2. We recommend using a 5 mL pipette for aspirations and resuspensions > 1 mL.
3. Record everything in **SHARE spreadsheet**.

Materials

	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
	1 M HEPES pH 7.3	Sigma	H0887-100ml
	NaCl	Fisher	BP358-1
	MgCl2	Fisher	AA12315A7
	Tween-20	Fisher	BP337-500
	5% digitonin	Millipore Sigma	300410
	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
	LS Columns	Miltenyi Biotec	130-042-401
	QuadroMACS Separator	Miltenyi Biotec	130-091-051
	Anti-Nucleus MicroBeads	Miltenyi Biotec	130-132-997

	Name	Manufacturer	Cat. #
	NucBlue Fixed Cell ReadyProbes	Thermo Fisher	R37606
	Disposable hemocytometer	Millipore Sigma	MDH-4N1-50PK

Buffers

1 Prepare the following buffers:

	Reagent	Amount	Final concentration
	Nuclei Extraction Buffer	35 mL	NA
	RNase inhibitor, murine	175 uL	0.2 U/uL

Lysis buffer

	Reagent	Amount	Final concentration
	Nuclei Extraction Buffer	35 mL	NA
	7.5% BSA	333 uL	0.1%
	RNase inhibitor, murine	125 uL	0.2 U/uL

RSB

	Reagent	Amount	Final concentration
	Nuclei Extraction Buffer	6.9 mL	14%
	PBS	41.4 mL	NA
	7.5% BSA	257.6 uL	0.04%

Nuclei Suspension Buffer (NSB)
Make 2 tubes of 50 mL.

	Reagent	Amount	Final concentration
	Nuclei Suspension Buffer (NSB)	30 mL	NA
	RNase inhibitor, murine	150 uL	0.2 U/uL

NSB-RI
Make 2 tubes of 30 mL.

	Reagent	Amount	Final concentration
	H2O	48.75 mL	NA
	1 M HEPES pH 7.3	500 uL	10 mM
	5 M NaCl	100 uL	10 mM
	1 M MgCl2	150 uL	3 mM
	RNase inhibitor, murine	500 uL	0.1%

HT-RSB

	Reagent	Amount	Final concentration
	HT-RSB	25 mL	NA
	7.5% BSA	133.8 uL	0.04%
	5% digitonin	50 uL	0.01%
	Enzymatics RI	62.5 uL	0.1 U/uL
	SUPERase RI	31.3 uL	0.025 U/uL
	Yeast tRNA	250 uL	100 ug/mL

HDT-2RI
Make 2 tubes of 25 mL.

	Reagent	Amount	Final concentration
	Glycine	0.94 g	2.5M
	H2O	5 mL	NA

2.5 M Glycine

Setup

2 Before starting, spray down the bench and pipettes with 70% ethanol and RNase-away.

3 Coat SHARE-seq nuclei prep tubes with BSA. Fill 1.5 mL tubes with 1.5 mL 1% BSA-DEPC and incubate for 30 minutes. After incubation, aspirate BSA solution and dry for 30 minutes. Store at 4°C. Alternatively, add 150 uL 7.5% BSA, vortex, remove supernatant, let tube sit on ice briefly, and aspirate BSA solution.

4 Pre-chill centrifuge to 4°C.

5 Prepare ice buckets.

6 Prepare **35 mL** lysis buffer in a 50 mL conical tube on ice. Distribute 2 mL into 8 gentleMACS C Tubes on ice.

7 Prepare **25 mL** RSB in a 50 mL conical tube on ice.

8 Prepare **two 25 mL** aliquots of HDT-2RI in 50 mL conical tubes. Keep one at room temperature and the other on ice.

9 Prepare **two 40 mL** aliquots of NSB in 50 mL tubes on ice.

10 Prepare a fresh 2.5M glycine solution (usable for up to one month). Weigh the glycine powder into a 5 mL tube, add water, and allow it to dissolve. If needed, incubate at 37°C

in a thermomixer.

11 Distribute NucBlue Fixed Cell ReadyProbes into PCR strip tubes for cell counting.

12 Print labels and label 1.5 mL tubes for final fixed pellets.

Tissue lysis and nuclei extraction

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

14 Drop whole frozen tissue into a chilled gentleMACS C Tube with **2 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.

15 Run the gentleMACS Program 4C_nuclei_1 on the Octo Dissociator (~5 minutes). Add chilled adapters to make sure nuclei stay cold.

16 Remove tubes, ensuring tissue did not get stuck on the sides, and spin down in a 4°C centrifuge for ~10 seconds to bring liquid to the bottom, then place tubes back on ice.

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

18 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.

19 Discard strainer and centrifuge the nuclei suspension at 4°C, 350g for 5 minutes.

20 Resuspend nuclei pellet in **3 mL RSB**.

21 Filter nuclei suspension through 30 um MACS SmartStrainer into a labeled 5 mL tube.

22 Count nuclei. Use 1:11 dilution factor, 20 uL dye + 2 uL sample.

Anti-Nucleus MicroBeads selection

23 Set aside 4 million nuclei in **RSB** in a new 5 mL tube and spin down at 4°C, 300g for 5 minutes.

24 Pipette off supernatant completely without disturbing the nucleus pellet

25 Resuspend nucleus pellet in **1.8 mL NSB-RI**.
(450 µL of nuclei separation buffer per 10^6 total nuclei)

26 Add **200 uL of Anti-Nucleus MicroBeads**
(50 µL of Anti-Nucleus MicroBeads per 10^6 total nuclei)

27 Mix well and incubate for 15 minutes in the refrigerator (2–8°C).

28 During incubation, prepare LS columns (need 1 column per sample):
Place column in the magnetic field of a suitable MACS Separator.
1. Label LS columns, 15 mL tubes for debris, and 5 mL tubes for nuclei.
2. Place LS columns in the magnetic separator rack and 15 mL tubes in the clear plastic tube holder.
3. Rinse the columns by passing **1 mL NSB** (no RI) 3 times (**3 mL total**) with a 10 mL serological pipette. Wait the first mL pass through, no dripping, then add the next mL

29 Add **2 mL NSB-RI to the samples** and proceed to magnetic separation with LS Columns.

30 Apply nucleus suspension onto the column, **1 mL at a time**. Collect flowthrough containing debris in the 15 mL tubes. Add the nucleus suspension directly to the center of the column reservoir. Always wait until the column reservoir is empty before proceeding to the next step.

31 Wash column **two times with 1 mL NSB-RI**. Collect debris that passes through and combine with the flow-through from the previous step. Perform washing steps by adding buffer aliquots as soon as the column reservoir is empty. Use buffer to rinse the inner column walls.

32 **Remove column from the separator** and place it on a **5 mL** collection tube. Typically just hold it over the 5 mL tube with one hand.

33 With the other hand, pipette **1 mL NSB-RI** onto the column. Immediately flush out the magnetically labeled nuclei by firmly pushing the plunger into the column.

34 Optionally, count nuclei to measure recovery after bead cleanup. Start the 5 minute spin in the next section and count then; if necessary, finish during the 10 minute incubation at step 4 in the next section. Use 1:4 dilution factor (9 uL dye + 3 uL sample).

SHARE-seq nuclei fixation

35 Spin down nuclei in the 5 mL collection tubes at 4°C, 750g for 5 minutes.

36 Remove supernatant and resuspend nuclei pellet in **4 mL** room temperature **HDT-2RI.** Transfer tube to a room temperature rack.

37 At RT, add **53.4 uL** of methanol-free formaldehyde in the fume hood (16% stock solution). Final concentration for nuclei: 0.2%. Close tube and nutate or rotate nuclei at RT for 5 minutes.

38 To quench fixation, per reaction, add **224.4 uL** fresh 2.5M Glycine, **200 uL** of 1M Tris pH 8.0, **53.2 uL** of 7.5% BSA, and mix using a pipette. Incubate on ice for 10 minutes.

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

40 Add **500 uL** of HDT-2RI and gently resuspend pellet. Transfer to labeled 1.5 mL tube. Store on ice.

a. Count nuclei at 1:10 dilution factor (18 uL dye + 2 uL sample)
b. For multiple aliquots, split 500 uL into 2 tubes of 250 uL.

41 Spin 750xg, 4°C, 10 minutes. Gently remove supernatant. Remove all fluid and freeze at -80C as a dry pellet.