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(P75) Isolation of single nuclei from frozen tissues V.1

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We are still developing and optimizing this protocol

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Disclaimer

Please note that this protocol is not peer-reviewed and is provided without warranty. Users should validate the method for their specific sample types by assessing RNA integrity and yield. Known issues of this protocol have been annotated as "CRITICAL" notes behind the steps. Not following these critical notes may result in undesirable results.

Abstract

This protocol details the preparation of cryopreserved samples for single-nucleus RNA sequencing (snRNA-seq) and the subsequent storage of extracted nuclei for future use. This workflow is particularly advantageous when fresh tissue access is limited or when longitudinal study designs require the reuse of the same nuclei batches.

Materials

UltraPure, 1M, pH 8.0, Tris-HCl
ThermoFisher, 15568025

Sucrose
Sigma S0389

KCl
Sigma P5405

1M MgCl2
ThermoFisher, AM9530G

10% Triton X-100
Sigma, 648463

RNasin Plus RNase Inhibitor
Promega, N2611

Protease inhibitor
Promega G6521

100 mM DTT
ThermoFisher, 707265

BSA
VWR, 0332

Ultrapure, H2O
ThermoFisher, 10977

Protector Rnase Inhibitor
Roche, 3335399001

10X PBS
ThermoFisher, 70011

Dounce homogenizer
Fisher, 06-434

Anti-NeuN Antibody, clone A60, Alexa Fluor 488 conjugated
Sigma, MAB377X

Kynurenic acid sodium salt
Hellobio, HB0363

NaH_2PO_4
Sigma, S5011

HEPES
Sigma, H3375

Sodium ascorbate
Sigma, A4034

Thiourea
Sigma, T7875

Sodium pyruvate
Sigma, P5280

$CaCl_2$
Sigma, C5670

Taurine
Sigma, T8691

Myo-inositol
Sigma, I5125

NMDG
Sigma, M2004

HCl
Sigma, 258148

APV
Sigma, A8054

DNQX
Sigma, D0540

TTX
Tocris, 1078

D-(+)-Trehalose dihydrate
Sigma, 90210

D-(+)-Glucose
Sigma, G7021

Sodium Bicarbonate 7.5% solution
ThermoFisher, 25080094

O.C.T.
Fisher Scientific, NC1862249

DMSO
ThermoFisher, D12345

pluriStrainer Mini 40 µm (Cell Strainer)
PluriSelect, 43-10040-60

pluriStrainer Mini 70 µm (Cell Strainer)
PluriSelect, 43-10070-60

Draq5
Biolegend, 424101

SiR-DNA
Cytoskeleton, CY-SC007

Troubleshooting

Before you start

45m

1 Prepare 50 mL NIM1 by following the table below. 5m

	A	B	C
	Reagent	Final concentration	Amount
	1M Tris pH8.0	10 mM	500 µL
	Sucrose	250 mM	4.26 g
	KCl	50 mM	0.1864 g
	1M MgCl2	5 mM	250 µL
	H2O	n/a	To 50 mL
	Total	n/a	50 mL

Note: This solution can be prepared in advance and stored at 4C for 1 month.

2 Prepare 100 mM taurine by following the table below.

	A	B	C
	Reagent	Final concentration	Amount
	Taurine	100 mM	0.625g
	H2O	n/a	To 50 mL
	Total	n/a	50 mL

Note: This solution can be prepared in advance and stored at -20C for 1 year.

3 Prepare 10% BSA by following the table below. 5m

	A	B	C
	Reagent	Final concentration	Amount
	BSA	10%	1 g
	H2O	n/a	To 10 mL
	Total	n/a	10 mL

Note: This solution can be prepared in advance and stored at -20C for 1 year.

4 Prepare 100X Kynurenic acid solution by following the table below. 5m

	A	B	C
	Reagent	Final concentration	Amount
	Kynurenic acid sodium salt	100 mM	1 g
	H2O	n/a	To 47.36 mL
	Total	n/a	47.36 mL

Note: Aliquot into 2mL each, store at -20C.

5 Prepare 25 mM APV by following the table below. 5m

	A	B	C
	Reagent	Final concentration	Amount
	APV	25 mM	50 mg
	H2O	n/a	To 10 mL
	Total	n/a	10 mL

Note: Aliquot into 200 µL each, store at -20C.

6 Prepare 100 mM DNQX by following the table below. 5m

	A	B	C
	Reagent	Final concentration	Amount
	DNQX	100 mM	50 mg
	DMSO	n/a	1.68 mL
	Total	n/a	1.68 mL

Note: Aliquot into 200 µL each, store at -20C.

7 Prepare 100µM TTX by following the table below. 5m

	A	B	C
	Reagent	Final concentration	Amount
	TTX	100 uM	1 mg
	H2O	n/a	31 mL
	Total	n/a	31 mL

Note: Aliquot into 200 µL each, store at -20C.

8 For vertebrates including mice, voles, lizards, frogs, and fish: 15m
Prepare 10X ACSF by following the table below.

	A	B	C
	Reagent	Final concentration (mM)	Amount (g)
	KCl	25	1.864
	NaH2PO4	12	1.44
	HEPES	200	47.66
	Sodium ascorbate	50	9.9
	Thiourea	20	1.522
	Sodium pyruvate	30	3.3
	CaCl2	10	1.11
	1 M MgCl2	13	13.17 mL
	100 mM Taurine	0.1	1 mL
	Myo inositol	30	5.4
	NMDG	960	187.4
	35% HCl	n/a	pH to 7.3
	H2O	n/a	To 1 L
	Total	n/a	1 L

Note: Store the 10X ACSF in 20 mL/tube aliquot at -20C. The thiourea turns yellow when ACSF expires.

For invertebrate, fruit fly:
Prepare 10X AHL by following the table below.

	A	B	C
	Reagent	Final concentration (mM)	Amount (g)
	KCl	50	3.728
	NaH2PO4	10	1.2
	HEPES	50	11.9
	Sodium ascorbate	50	9.9
	Thiourea	20	1.522
	Sodium pyruvate	30	3.3
	CaCl2	20	2.22
	1M MgCl2	82	83 mL
	100 mM Taurine	0.1	1 mL
	Myo inositol	30	5.4
	NaCl	1000	58.44
	35% HCl	n/a	pH to 7.5
	H2O	n/a	To 1 L
	Total	n/a	1 L

Note: Store the 10X AHL in 20 mL/tube aliquot at -20C. The thiourea turns yellow when AHL expires.

Prepare solution.

22m

9 For vertebrates including mice, voles, lizards, frogs, and fish:
Prepare 1X ACSF by following the table below in order.

10m

	A	B	C
	Reagent	Final concentration (mM)	Amount (g)
	H2O	n/a	150 mL
	10X ACSF	1X	20 mL
	Glucose	20	0.9 g

	A	B	C
	Trehalose	73	5 g
	25 mM APV	0.025	200 µL
	100 µM TTX	0.1	200 µL
	100 mM DNQX	0.1	200 µL
	100 mM Kynurenic acid	1	2 mL
	H2O	n/a	to 200 mL
	Total	n/a	200mL

CRITICAL: This solution needs to be prepared fresh. The pH should be arround 7.3. Osmolarity should be arround 300-310 mOsm.

For invertebrates, fruit fly:
Prepare 1X AHL by following the table below in order.

	A	B	C
	Reagent	Final concentration (mM)	Amount (g)
	H2O	n/a	150 mL
	10X ACSF	1X	20 mL
	Sucrose	10	0.685 g
	Trehalose	5	0.378 g
	25 mM APV	0.025	200 µL
	100 µM TTX	0.1	200 µL
	100 mM DNQX	0.1	200 µL
	100 mM Kynurenic acid	1	2 mL
	H2O	n/a	to 200 mL
	Total	n/a	200mL

CRITICAL: This solution needs to be prepared fresh. The pH should be arround 7.5. Osmolarity should be arround 260-280 mOsm.

10 Place the solution on ice. 1m

11 Oxygenate the solution with carbogen (95% O_2, 5% CO_2) for 5 minutes prior to the next step, maintaining continuous bubbling thereafter. 5m

12 For vertebrates including mice, voles, lizards, frogs, and fish:
Add 6mL 7.5% $NaHCO_3$.
For invertebrates, fruit fly:
Add 896 µL 7.5% $NaHCO_3$. 1m

13 Add ddH2O to 200mL.
Note: the prepared solution is kept on ice with continuous oxygenation through the entire experiment. 5m

Tissue dissection 33m

14 Sacrifice the animal using CO2 and cervical dislocation. 15m

15 Dissect the brain in ice cold ACSF/AHL prepared in step 12.

16 Embed into O.C.T.. 5m

17 Float a PCR rack on liquid nitrogen. 1m

18 Place the O.C.T. block on the PCR rack.
Critical: Placing the O.C.T. block directly into the liquid nitrogen will break the block. 1m

19 Wait until the O.C.T. block completely freezes. 10m

20 Store the snap frozen tissue under -80C. 1m

Nuclei extraction 2h 38m

21 Prepare NIM2 by following the table below. 2m

	A	B	C
	Reagent	Final concentration	Amount
	NIM1	1 X	4.9 mL
	100 mM DTT	0.1 mM	5 µL
	50X protease inhibitor	1 X	100 µL
	10% BSA	0.1%	50 µL
	Total	n/a	5 mL

22 Prepare NIM3 by following the table below. 2m

	A	B	C
	Reagent	Final concentration	Amount
	NIM2	1 X	1 mL
	Protector RNAse inhibitor/or RNasin plus	0.2 unit/µL	5 µL
	Total	n/a	1 mL

Note: 1 mL of NIM3 is used per sample.

23 Prepare nuclei freezing medium by following the table below. 2m

	A	B	C
	Reagent	Final concentration	Amount
	NIM2	90%	900 µL
	Protector RNAse inhibitor/or RNasin plus	0.2 unit/µL	5 µL
	DMSO	10%	100 µL
	Total	n/a	1 mL

24 Place the Dounce homogenizer (Dounce for short from here on) on ice. 1m

25 Add 1mL NIM3 to a Dounce. 1m

26 Cryosection the O.C.T. block into 200 um sections. 1h

27 Keep the section cold and place the section onto dry ice plate. 1m

28 Dissect the region out while the section is frozen under a microscope. 1h

29 Transfer the dissected tissue into the Dounce on ice. 2m
Critical: the amount of tissue should be limited. Too much tissue at this step will result in failed extraction. A general starting point is 50mg in weight or 2mm in size.

30 Dounce on ice with loose pestle 10 times, 1m

31 Dounce on ice with tight pestle 5 times. 1m
CRITICAL: For tissue type used the first time, the number of Dounce should be determined by a few trial experiment. Optimal number is determined by generating the largest amount of nuclei while maintaining the integrity of the nuclei morphology.

32 Centrifuge 400g, 4C, 5min. 5m

33 Remove supernatant. 1m

34 Add 100 µL of 10% BSA into 900 µL of NIM3 to make 1%BSA cushion. 1m

35 Transfer the 1 mL 1%BSA cushion into a 5mL polypropylene flow tube. 1m

36 Add 10 µL 10% Triton X-100 to 1mL NIM3, mix gently to avoid bubbles. 1m

37 Use the NIM3 with tritonx100 to slowly resuspend pellet by pipetting up and down 3 times. 1m

38 On ice 1 minute. 1m

39 Filter through 70 µm filter. 1m

40 Filter through 40 µm filter. 1m

41 Carefully lay the cell lysate on the top of the cushion. 1m

42 Centrifuge 400g, 4C, 5min. 5m

43 Resuspend the pellet into 1mL nuclei freezing medium. 1m
Optional: check the nuclei quality and quantity here. Good quality nuclei should look round, with minimal visible tissue debris, without small membranes, and abundant in quantity. Most if not all of the nuclei should be stained with cell nonpermeable dyes.

44 Aliquot into 10-20 tubes depending on the need. 5m

45 Store the aliquots at -80C.

Nuclei staining, selection of neuronal nuclei, and FANS purification 2h 5m

46 Prepare nuclei staining buffer by following the table below. 5m

	A	B	C
	Reagent	Final concentration	Amount
	10X PBS	1X	250 uL

	A	B	C
	10% BSA	1%	250 uL
	ddH2O	n/a	2000 uL
	Protector RNAse inhibitor/or RNasin plus	0.2 unit/uL	12.5 uL
	Total	n/a	2.5 mL

47 Thaw the DMSO stored samples on ice. 10m

48 Add 1000 µL staining buffer and gently mix by slowly pipetting up and down. 1m

49 Centrifuge 400g, 4C, 5min. 5m

50 Discard supernatant. 1m

51 Resuspend in 200 µL staining buffer. 1m

52 Add 1 µL NeuN antibody. 1m

53 4C incubate 20 minutes. 20m

54 Add 800 µL staining buffer. 1m

55 Centrifuge 400g, 4C, 5min. 5m

56 Discard supernatant. 1m

57 Resuspend in 500 µL staining buffer

1m

58 Add 0.5 µL 1000X SiR-DNA or Draq5. 1m

59 Filter through 40 µm filter. 1m

60 Briefly examine quality by mixing with propidium iodide and trypan blue. 10m

61 Wet a 1.5 ml tube with 18.8 µl RT reagent B. 1m

62 Sort 24000 cells using 70µm nozzle and lowest sample pressure (=43.3 µl) into 18.8 RT mix. For bigger nuclei (e.g. frog), 18000 nuclei using 100µm nozzle (= 43.3 µl) into 18.8 RT mix.
CRITICAL: One should try to use the lowest possible sorting pressure possible to avoid breaking the nuclei during the sorting. 1h

63 Proceed to 10X droplet generation.