Village Nuclei Isolation With Optiprep V.3

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MANUSCRIPT CITATION:
Wells et al., PMID 36796362

ABSTRACT

Isolation of nuclei from fresh-frozen brain tissue from sets of multiple (typically 2-20) human donors for analysis as a “cell village” (**Wells et al., PMID 36796362**) in which nuclei from all donors are analyzed together. Adapted from dx.doi.org/10.17504/protocols.io.bs99nh96, Luciano G Martelotto, with modifications to optimize for human brain tissue and allow the “cell village” approach.

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

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Keywords: fresh-frozen nuclei, nuclei extraction, snRNAseq, RNAseq, human tissue, brain tissue, multiple donors

MATERIALS

Supplies

- Scalpels
- Glass slides
- Dounce
- 20 µm vacuum filter
- Eppendorf tubes (1.5 mL and 5 mL)
- Eppendorf or Ranin pipette tips
- Dry ice
- Metal plate
- OCT (Optimal cutting temperature compound)
- RNAse free water
- Cell counting supplies (LUNA-FL)

Other Reagents:

- PBS
- BSA
- RNAse inhibitor (i)
- Nuclei EZ lysis buffer (NUC201-1KT)
- OptiPrep Density Gradient Medium (60% Iodixanol - G60) (ab286850)

Stock solutions:

- **500 mM tricine**: 8.96 g of tricine in 100 mL water
- **1 M KCl**: 7.45 g of KCl in 100 mL water
- **1 M MgCl2x6H2O**: 20.3 g of MgCl2x6H2O in 100 mL water

BEFORE START INSTRUCTIONS

- Turn on and chill centrifuge to 4°C
- Prepare all the reagents needed on ice
- Clean glass slides with ethanol
- Gather the dounce, pestles, and scalpels on ice

Solutions to make fresh before starting experiment:

- Nuclei EZ lysis buffer -- Pour 12 mL of buffer into the dounce and reserve 1 mL for wetting down the sides of the dounce
- PBSA + i (1% BSA and 1 U/µL RNase inhibitor in 1X PBS) -- For powder BSA, add 0.01 g of BSA per 1 mL of 1X PBS
- G30: (Mix the G60 and GD together thoroughly before adding the GH)

Before Starting

1 Gather Supplies

- Scalpels
- Glass slides

- 14 mL Dounce
- 20 µm vacuum filter
- Eppendorf tubes (1.5 mL and 5 mL)
- Eppendorf or Rainin pipette tips
- Dry ice
- Metal plate
- OCT (Optimal cutting temperature compound)
- RNAse free water
- Cell counting supplies (LUNA-FL)

Other Reagents:

- PBS
- BSA
- RNAse inhibitor (i)
- Nuclei EZ lysis buffer (NUC201-1KT)
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Stock solutions:

- **500 mM tricine**: 8.96 g of tricine in 100 mL water
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1.1 Optiprep Buffers (can be made in advance and kept on hand)

	A	B	C	D
	GD		GH	
	Component	Volume	Component	Volume
	500 mM Tricine	24 mL	Sucrose	8.5 g
	1M KCl	15 mL	Water	50 mL
	1M MgCl2 x 6H2O	3 mL	500 mM Tricine	4 mL
	Water	50 mL	KCl	2.5 mL
	1M KOH	Adjust to pH 7.8	1M MgCl2x6H2O	0.5 mL
		Bring volume up to 100 mL with water (keep at room temp)	1M KOH	Adjust to pH 7.8
				Bring volume up to 100 mL with water (keep at 4°C)

1.2

- Turn on and chill centrifuge to 4°C
- Prepare all the reagents needed on ice
- Clean glass slides with ethanol

- Gather the dounce, pestles, and scalpels on ice

1.3 Solutions to make fresh before starting experiment

Nuclei EZ lysis buffer

- Pour 12 mL of buffer into the dounce and reserve 1 mL for wetting down the sides of the dounce

PBSA + i (1% BSA and 1 U/µL RNase inhibitor in 1X PBS)

- For powder BSA, add 0.01 g of BSA per 1 mL of 1X PBS

G30: (Mix the G60 and GD together thoroughly before adding the GH)

	A	B	C	D	E	F
	G30		PBSA + i		Nuclei EZ Lysis Buffer	
	Component	Volume	Component	Volume	Component	Volume
	G60 (OptiPrep)	6.0 mL	PBS (pH 7.4) (1X)	1 mL of 10X PBS	EZ Lysis Buffer	12,975 µL
	GD	1.2 mL	BSA (100X)	1 mL of 10% BSA	RNAse Inhibitor	325 µL
	GH	4.8 mL	RNase Inhibitor (200X)	50 µL		
			RNase free water	7,950 µL		

Tissue Homogenization

1h 30m

2 On a glass slide **on dry ice**, shave off pieces of tissue with a scalpel until you have 50±3 mg of tissue

2.1 For managing tissue, use OCT to adhere tissue to glass slide

3 Place tissue pieces on a boat on dry ice. Hold the tissue on dry ice until all tissue has been cut

4 Transfer all tissue to dounce filled with Nuclei EZ Lysis Buffer

4.1 Use 1mL of the reserved Nuclei EZ Lysis Buffer to wet down the side of the dounce if tissue is stuck on the side of the dounce

5 Gently dounce tissue on ice 20 times with pestle “A” then 20 times with pestle “B” until the tissue is homogenized

6 Incubate on ice for 10 minutes

Nuclei Isolation

1h 30m

7 Wet a 20 µm vacuum filter with 1 mL of PBSA + i

1h

8 Vacuum filter the homogenized tissue

8.1 If the filter clogs, remove any unfiltered volume with a pipette, replace the filter, and continue filtering

9 Transfer the homogenized tissue to (3) 5 mL tubes

10 Centrifuge the samples at 4°C for 5 min (500 x g)

11 Remove and discard the supernatant (be careful not to disturb cell pellets) and resuspend each pellet in 900 µL G30

11.1 Alternatively, split pellet into (9) 1.5 mL tubes (total), resuspend each pellet in 300 µL of G30, then underlay each sample with 1 mL of G30

12 Gently underlay each 5mL tube with 3 mL G30

Place the pipette tip filled with G30 at the bottom of the 5mL tube. Slowly dispense G30 underneath the cell suspension. A clear separation will form between homogenate and G30 (cloudy on top, clear on the bottom). Do not disturb the separation between the layers

13 Centrifuge the samples at 4°C for 10 min (4,255 x g)

13.1 For capturing smaller cell types, spin at 8,000 x g for 20 minutes

14 Remove and discard the supernatant

14.1 Remove the top (very viscous) layer from the tube first- this layer contains fats and must be removed before removing the rest of the supernatant

Nuclei Wash, Resuspension, and Quantification

20m

15 Resuspend the pellets in 1 mL PBSA + i

16 Centrifuge the samples at 4°C for 5 min (500 x g) (single wash)

17 Remove the supernatant and resuspend the pellets in 200 - 1,000 µL of PBSA + i (depending on the size of the pellet)

18 Count nuclei using LUNA-FL counter

Preserving Leftover Nuclei

15m

19 Optional if you have excess nuclei, or if you need to pause before continuing.

20 Centrifuge the nuclei at 4°C for 5 min (500 x g)

21 Remove the supernatant and resuspend the nuclei in CryoStor (CryoStor® CS10), mix by pipetting

21.1 ≤ 1k nuclei / µL of CryoStor

22 Transfer the nuclei to cryotubes and store them in a Mr Frosty in a -80°C freezer until frozen (1 day)

23 For long term storage, transfer cryotubes to regular storage in a -80°C freezer

Thawing Cryo-Stored Nuclei

30m

24 Thaw the CryoStored nuclei

25 Centrifuge the nuclei at 4°C for 10 min (1200 x g)

26 Remove the supernatant and resuspend in 1mL PBSA + i

27 Centrifuge the nuclei at 4°C for 5 min (500 x g) (single wash)

28 Remove the supernatant and resuspend the pelleted nuclei in PBSA + i

28.1 Count the nuclei before proceeding to next steps