Extraction of Nuclei from Brain Tissue

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1 Works for me

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ABSTRACT

This protocol enables rapid isolation of intact nuclei from brain tissue, including highly myelinated adult brain tissue. We have used it successfully to extract nuclei for single-nucleus RNA-seq from human, mouse, marmoset, and macaque brain.

ATTACHMENTS

Protocol for Nuclei Extraction.docx

GUIDELINES

As general principles, working quickly and keeping things cold will help ensure the best results.

MATERIALS TEXT

Wash Buffer (WB):

Reagent	For 500 mL	For 1L
Na_2SO_4	5.8 grams	11.6 grams
K_2SO_4	2.6 grams	5.2 grams
Glucose	0.9 grams	1.8 grams
HEPES	1.2 grams	2.4 grams
1M $MgCl_2$	2.5 mL	5 mL

Wash Buffer Recipe

1. Dissolve in water, bring up to the desired volume, and pH to 7.4 with 10N NaOH.
2. Measure osmolarity (acceptable range is 295-310 Osm/L).
3. Make ahead of time and store at 4°C.

This buffer was originally developed for dissociating neurons and described in:
Carter BC, Bean BP. Sodium entry during action potentials of mammalian neurons: incomplete inactivation and reduced metabolic efficiency in fast-spiking neurons. *Neuron*. 2009;64(6):898–909. doi:10.1016/j.neuron.2009.12.011

WB + 5% Kollidon VA64:

Place 5 grams of Kollidon VA64 for every 100 mL WB into a bottle containing a stir bar. Cap loosely. Stir slowly until the Kollidon has dissolved completely. This can take several hours. Periodic shaking (with cap tightened) will speed the process. Do not spin too quickly or over night, as this can cause the solution to turn cloudy (if it does so, discard). Store at 4°C.

Nuclear Extraction Buffer (NEB):

Make on the day of the extraction. 1% Triton X-100 in WB+5% Kollidon VA64. 3-5 mLs of NEB is required per sample. Allow the WB+5% Kollidon VA64 to come to room temperature before adding the Triton X-100 (otherwise it will not go into solution easily). Add Triton and pipette up and down rapidly to mix. Vortex and then place on ice when fully incorporated.

For each sample you wish to extract you will need the following:

- Clean glass slide and new razor blade, at 4°C
- Dissecting tray, kept at -20°C

- Ruler
- 3 mL syringe with 26 ½ gauge needle, at 4°C (have a few extra chilled ones on hand in case the first one clogs)
- 4 mL chilled extraction buffer, placed in first well of 6-well plate
- 6-well tissue-culture plate, well-bottoms colored, on ice
- ~50 mL chilled DB
- Diluted Hoechst stain (4 uL Hoechst + 996 DB)
- 20 µm Steriflip filter + tube, on ice
- 5 µm Pluriselect (yellow) filter plus adaptor (green) + 50 mL tube, on ice
- 4 50 mL tubes, on ice
- c-Chip Fuchs Rosenthal hemacytometer

BEFORE STARTING

Ensure that there is enough Wash Buffer (WB) and WB + 5% Kollidon VA64 on hand ahead of time. Organize all consumables prior to extraction and place on ice. Keep tissue at -80°C until sampling and return to -80°C immediately after sampling to minimize thawing and to preserve the tissue for future use. Move as quickly as is safely possible during the extraction to reduce mRNA degradation.

Prior to extraction:
Place 50 mL adaptors into centrifuge buckets and set temp to 4°C so it will be cold when you are ready for it.
Set up all needed buffers and equipment so you can move as smoothly and quickly as possible through the extraction.

1 Retrieve dissection tray from -20°C, and the glass slide and razor blade from 4°C.

2 Place the glass slide, razor blade, and ruler on the dissection tray and perform all cutting on slide to minimize damage to, and contamination of, the tray.

It is helpful to put the dissecting tray on ice to keep it cold and prevent it from moving.

3 Place the frozen sample on the glass slide and shave off the frosty/freezer-burned tissue from the face you want to sample from, and discard. This is damaged and not worth extracting. Then, moving rapidly but carefully, shave off enough sample to work with.

4 Return the tissue to -80°C (or put on dry ice as a temporary hold).

5 Rapidly mince the shaved sample with the same razor blade that was used to shave the tissue in step 3 and scrape into the first well of a 6-well plate that contains 4 mL of cold extraction buffer. Pipette up and down 20 times.

6 Commence 10-minute incubation, pipetting up and down 20x every ~2.5 minutes with a 1000 uL pipette. **4 °C**

7 Pull the sample into the chilled needle/syringe and express (expel) into the second well of the 6-well plate.

8 Repeat this, pulling the sample into the same syringe and expressing it into the third well of a 6-well plate.

Note that one may simultaneously process 2 distinct samples in the top and bottom 3 wells of a 6-well plate.

9 Wet the 20 µm filter with 200 uL cold WB, then pull the sample through the filter using gentle vacuum. Then pass 2 mL of cold WB through the filter as a wash step.

Try to avoid frothing (bubbles).

10 Optional Step: Wet the 5 µm filter with 200 uL WB and put the sample through that filter, using gentle vacuum. Try to avoid frothing. Then pass 2 mL of cold WB through the filter as a wash step.

Using the 5 µm filter may not be necessary if you are working with a sample that has a low amount of debris/white matter, or if you do not want to risk filtering out some of the larger nuclei (although most of the larger nuclei will still squeeze through the 5 µm filter with the vacuum).

11

Optional Step: Remove 10 uL of nuclei and mix with 10 mL of the diluted Hoechst stain and view using the brightfield and DAPI filters on the fluorescent scope. Photograph both brightfield and DAPI-excited nuclei.

This is your baseline extraction and it's a check to see if you have nuclei (and if it is worth proceeding with the prep). You will still see debris, despite the filtration steps. The subsequent spin will remove much of the debris, though some will still pellet with the nuclei.

It is helpful to have a second person perform this step while you continue with step 12.

Once you are familiar with extracting nuclei from a particular type of tissue and are consistently getting the expected nuclei yields, this baseline check step can be skipped.

12 Add chilled WB to the filtered nuclei to a total volume of just under 50 mL.

13 Divide these diluted nuclei among four 50 mL conical tubes (~14 mL per tube).

Don't use 15 mL tubes as the nuclei do not "travel" the longer distance well.

14 Spin at 500 rcf for marmoset and human nuclei (600 rcf for mouse nuclei) for 10 minutes at 4°C. **00:10:00**

00:10:00

4 °C

15 Carefully remove as much of the supernatant as possible without disturbing the pellet.

The pellet is hard-to-impossible to see. The cleaner the prep, the less visible the pellet is.

16 Resuspend the pellet in ~1 mL of WB, serially combining the pellets from all 4 tubes.

You will end up with ~1.5 - 2 mLs of extracted nuclei (the 1 mL you added plus the remaining WB that you had to leave in the tube to not disturb the pellet).

17 Add 10 mL nuclei + 10 mL diluted Hoechst stain and visualize with the fluorescent scope, getting images of brightfield and DAPI-excited nuclei.

It is normal to see some debris but you should see less than what you saw in the baseline visualizations from step 11.

18 Obtain nuclei counts, calculate concentration, and dilute to the desired concentration using DB.

For Nuclear Drop-seq, we typically dilute to 176 nuclei/uL.