Tissue Dissociation and Nuclei Isolation

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1 Works for me dx.doi.org/10.17504/protocols.io.bes7jehn

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ABSTRACT

This protocol details a step in the workflow for our contribution to the BRAIN Initiative Cell Census Network (BICCN) – Comprehensive Center on Mouse Brain Cell Atlas (U19) project. In previous steps of the workflow, we collected flash-frozen tissue from select areas of the murine cortex. Here, we dounce homogenize and digest the tissue in order to isolate the nuclei into a PBS, BSA, and RNase inhibitor solution, which is then FACS sorted to isolate nuclei subpopulations of choice. The protocol is adapted from Habib et al., *Massively parallel single nucleus RNA-seq with dronc-seq,* Nature Methods, 2017, with additional commentary by Eugene Drokhlynasky and Ehsan Habibi of the Regev lab. Per Gene, this method for nuclei extraction works best; Per Ehsan, this method works very well with mouse brain tissue.

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Adapted from Habib et al., *Massively parallel single nucleus RNA-seq with dronc-seq,* Nature Methods, 2017.

ATTACHMENTS

nuc_prep and FACS for 10X v1.3 SCAN.pdf

Nuclear prep and FACS for 10x Genomics Chromium v1.3.2 5.2019.docx

MATERIALS

NAME	CATALOG #	VENDOR
Nuclei EZ lysis buffer	EZ PREP NUC-101	Sigma
Gibco™ (Phosphate Buffered Saline) Solution, pH 7.4 (PBS)	10010-049	Fisher Scientific
Vybrant™ DyeCycle™ Ruby Stain	V10309	Thermo Fisher
KIMBLE 2mL Glass Dounce Tissue Grinder Set	D8938	Sigma
Corning™ Falcon™ Test Tube with 35µm Cell Strainer Snap Cap	352235	Corning
BSA Molecular Biology Grade	B9000S	New England Biolabs
Recombinant Ribonuclease Inhibitor	2313A	Takara

BEFORE STARTING

Maintain RNAse free conditions throughout. Keep nuclei ice-cold at all times.

Preparation 19m

1 Prepare materials and reagents for the experiment. If there alre multiple people carrying out the experiment, Steps 1.2, 1.3, and 1.4 can be done while someone else carries out Step 2. Step 3 onward will require the NSB (Step 1.2) and NSB+Ruby (Step 1.3) solutions.

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1.1 10m
1. Pre-chill the centrifuge to 4°, set at 500 g for 5 min, turn acceleration and deceleration to < 3.
2. Retrieve the tissue samples, and keep them in dry ice until you are ready to begin the experiment.
3. Place a 5 mL centrifuge tube on ice for each sample.
4. Set aside 8 mL of EZ Lysis Buffer per sample.
5. Wash dounce and pestles with 100% EtOH, followed by RNAse Zap. Wash in 2-3 rounds of RNAse-free water, and rinse with EZ Lysis Buffer.
6. Chill dounce and pestles on ice (can place the dounce and pestles on saran wrap).

1.2 2m
Prepare ~5 mL Nuclei Suspension Buffer per sample. For 5 mL:
- 5 mL PBS
- 25 uL BSA (20 mg/mL)
- 25 uL RNase inhibitor (40 U/uL)

Place the NSB on ice.

1.3 4m
If using the Vybrant DyeCycle Ruby stain, make 1mL NSB and Ruby at 1:500 dilution per sample. Add an additional 5 uL / mL RNAse Inhibitor to this solution (final: 0.4 U/uL), because the nuclei will be sitting in it for a while.

Note: the Ruby is provided in a DMSO solution and is not very soluble in cold media. If necessary, dilute it 1:10 in a small amount of room-temperature NSB, then 1:50 into the cold NSB (final: 1:500). We use this cell cycle indicator instead DAPI - the conventional wisdom in Regev lab: DAPI inhibits reverse trasncriptsase and leads to lower quality sequence reads. Ruby is a far-red dye that shouldn't interfere with GFP fluoresence during sorting. Depending on what you are sorting on, you might want to modify this step.

Note: clumping of nuclei might indicate the need for a higher concentration of BSA in the final resuspension buffer. Per the 10X Genomics single-nucleus protocol, BSA can go up to ~1%.

1.4 3m
Prepare 25 uL of rich-NSB per sample to sort the nuclei into. For 50 uL:
- 50 uL NSB
- 2.5 uL RNAse Inhibitor (final: 2 U/uL)
- 2.5 uL BSA (final: 1%)

Keep the rich-NSB on ice until FACS sorting.

Plan to sort into 200 uL wells (e.g.: an 8-well PCR strip or a 96-well plate). Pick one well per sample and coat the entire inside with undiluted BSA. Place the plate in a clean ziplock bag to transport it to/from the flow cytometry core. (The BSA coating reduces adherence and improves recovery).
- Right before sorting, draw off the BSA in the wells and winse the well with NSB (the basic one), then fill with 10-20 uL of the rich-NSB, based in the volume you expect to collect - try to keep the final post-sort volume under 30 uL. Try to make sure there are no bubbles. The final post-sort volume will depend on the FACS sort nozzle size and expected number of sorting events.

Tissue Digestion 29m 30s

2 Dounce homogenize the tissue samples and digest it with the EZ Lysis Buffer. Digestion will consist of two cycles of incubation on ice with the EZ Lysis Buffer followed by centrifugation.

Note: the number of pestle strokes (Step 2.2) and incubation times (Steps 2.3 and 2.5) may need optimization based on the tissue. If too many nuclei have adherent cell material, try increasing; if there is excessive damage, try decreasing. (See EZ Prep kit documentation for additional troubleshooting.)

2.1 30s
Transfer tissue to chilled doucer. **Do not allow the tissue to thaw.** Immediately add 2 mL of ice-cold EZ Lysis Buffer.

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2.2 Homogenize with 25 strokes of pestle A and 20 strokes of pestle B. Raise and lower the pestles slowly, avoiding bubbles. **Keep the tissue on ice at all times.** 5m

2.3 Transfer the suspension to the 5 mL centrifuge tube (Step 1.1). Rinse douncer with 2 mL ice-cold EZ Lysis Buffer; tansfer rinsate to tube and gently mix by inversions. Incubate for 5 min. 6m

2.4 Centrifuge at 500 g for 5 min at 4°, with low acceleration and deceleration. 6m

2.5 Carefully discard the supernantant and resuspend the pellet in 4 mL ice-cold EZ Lysis Buffer by gentle pipetting. Incubate on ice for 5 min. 6m

2.6 Centrifuge at 500 g for 5 min at 4°, with low acceleration and deceleration. 6m

NSB Wash 7m

3 Carefully discard the supernatant and resuspend the pellet in 4 mL ice-cold Nuclei Suspension Buffer (NSB, Step 1.2). Resuspend by gentle pipetting. Immediately transfer to centrifuge. 1m

3.1 Centrifuge at 500 g for 5 min at 4°, with low acceleration and deceleration. During the centrifugation, follow Step 3.2 6m

3.2 For each sample, pre-wet a 35 um Cell Strainer Cap with ~150-200 um of NSB. Keep the filter tube on ice.

Nuclei Resuspension and Filtration 6m

4 Carefully discard the supernatant and resuspend the pellet in 1 mL ice-cold NSB+Ruby (Step 1.3). Resuspend by gentle pipetting. 1m

For large pieces of tissue, you can resuspend in ~1.2 mL NSB+Ruby so as to keep the nuclei and debris concentration within an acceptable range for the FACS machine.

4.1 Filter the nuclei suspension through the 35 um Cell Strainer Cap (Step 3.2) by slowly pipetting the suspension on top of the filter. **Do not force the suspension through the filter.** 2m

4.2 If the filter becomes clogged, mix the filtered suspension with the remaining unfiltered suspension. Pre-wet a new filter tube (Step 3.2) and filter again. 2m

This step is recommended as the tissue for larger pieces of tissue, as you risk clogging the FACS machine with any unfiltered debris that gets through.

5 Take the suspension and FACS sorting materials to the flow cytometry core for FACS sorting: 1m
- Nuclei suspension sample(s)
- 96-well plate or 8-well PCR strip, with BSA (Step 1.4)
- rich-NSB (Step 1.4)
- Remaining NSB (to rinse the wells of BSA)
- Pipettes, pipette tips, and filled ice bucket(s).

Proceed to the FACS sorting protocol.

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