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Neuron dissociation protocol for single-cell sequencing applications

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We use this protocol and it's working

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Disclaimer

This protocol was adapted from the work of Boxun Li in the Gersbach lab at Duke University.

Abstract

This protocol describes methods for neuron dissociation in preparation for single-cell sequencing applications.

Materials

- 1x Dulbecco's (D) PBS (DPBS) (no Ca2+/Mg2+, Thermo Fisher, 14190-144)
- Dissociation solution: Papain (>=20U/mL) in Accutase with 10µM ROCK inhibitor (ROCKi).
  - Papain: Worthington, LK003178
  - Accutase: Millipore, SCR005
  - ROCK inhibitor: Stemcell Tech, 72304
- DNase I (Worthington, LK003170): Make stock aliquots at 12500U/mL.
  - DNaseI should be thawed right before the experiment.

Maturation Medium	Neurobasal™-A Medium	50%	48.5 mL
	DMEM/F-12, HEPES	50%	48.5 mL
	NEAA (100X)	1X	1 mL
	B-27™ Supplement (50X)	0.5X	1 mL
	N-2 Supplement (100X)	0.5X	0.5 mL
	GlutaMAX™ Supplement (100X)	0.5X	0.5 mL
	BDNF	10 ng/mL	Add before use
	NT-3	10 ng/mL	Add before use
	Laminin	1 µg/mL	Add before use

Neuronal Maturation Medium (same as in protocol 1.Ngn2 neuron differentiation)

- Resuspension Medium: 49mL Maturation Medium + 1mL DNaseI stock supplemented with 10µM ROCKi.
  - Warm up
  - Add DNaseI right before trituration after 40-m incubation
  - Prepare 5mL per 10cm dish for trituration
- Maturation Medium + 1% BSA
  - 45mL Maturation Medium + 5mL 10% BSA solution (Sigma-Aldrich, A1595)

1 Prepare reagents
Prepare dissociation solution (Accutase/papain/ROCKi) right before when cells are ready:
- 1mL/well dissociation solution is needed per well for 6-well plates. Scale accordingly to other vessels.
- Dissolve each vial of papain with 5mL warm Accutase.
- To every 5mL of Accutase/papain solution, add 5µL ROCKi.

Put an aliquot of enough Maturation Medium in water/bead bath to warm up.

2 Aspirate medium from neurons. Wash once with 1x DPBS. Aspirate DPBS.
Note: Use 1mL/well DPBS for 6-well plates. Scale accordingly for different vessels.

3 Gently add dissociation solution to neurons without disturbing it. Use the same amount as DPBS.

4 Incubate the neurons at 37 °C for 40 min.
- Near the end of the incubation, add DNaseI and ROCKi to warm Maturation Medium to make Resuspension Medium. 5mL is needed for each full 6-well plate or 10cm dish. Aliquot 5mL of Resuspension Medium to each 15mL conical tube for a number of tubes, determined by the number of samples.

5 At the end of the incubation, neurons should easily come off the cell culture vessel as a sheet by rocking the vessel. Very **gently**, using a 10mL serological pipette, transfer the cell sheet(s) to a 15mL tube already containing 5ml Resuspension Medium, leaving behind as much dissociation solution in the vessel as possible.

6 Gently triturate the content of each tube 50x using a P1000 pipette to dissociate cell sheets into single cells.
Note:
- First 10 passes need to be slow to avoid over-shredding the cells. It will feel easier after 10 passes.
- Sufficient dissociation is critical at this step. It is recommended to check the cell suspension under a microscope and if necessary, pipette the cell suspension up and down more until the majority becomes single cells. However, over-pipetting could result in poor viability.

7 Pool contents of tubes together after pipetting, if desired.

8 The protocol diverges here depending on the downstream application (flow cytometry or scRNA-seq)

8.1 For flow cytometry:

- Centrifuge cells at **300xg for 5 minutes**.
- Resuspend cells in DPBS + 1% BSA.
- Cells are ready for flow cytometry.

8.2 For bulk ATAC-seq:

- Centrifuge cells at **300xg for 5 minutes**.
- Resuspend cells in freeze media (90% KnockOut Serum Replacement (Gibco, 10828010) + 10% dimethyl sulfoxide (Sigma-Aldrich, D8418)).
- Freeze cells at -80C in Mr. Frosty Freezing Container (Thermo Scientific, 5100-0001).
- Transfer frozen vials to liquid nitrogen for storage until ready for ATAC-seq.

8.3 For scRNA-seq:

Centrifuge cells at **300xg for 5 minutes**.

- Add 5mL/tube Resuspension Medium. Pipette triturate **gently** 20x using P1000.
- Centrifuge cells at **300xg for 5 minutes**.
- Add 1mL/tube Maturation Medium + 1% BSA. Pipette triturate **gently** 20x using P1000. Transfer cell suspensions to 1.7mL Eppendorf tubes.
- Centrifuge cells at **300xg for 5 minutes** with the table-top centrifuge, discard supernatants, and resuspend each pellet with 1mL Maturation Medium + 1% BSA with 20x **gentle** trituration by P1000 after each spin. Repeat this for a total of 3 times.
- After the second spin and resuspension, take 10µL to count each sample on a hemocytometer with Trypan blue.
- After the third spin, resuspend cell pellets thoroughly 30-50x by pipetting the cells gently up and down with P1000 in MM+1% BSA to a target concentration of 1500 cells/µL based on the previous count. It is expected that the actual concentration is lower than the target due to cell loss during the spin and straining steps below.
- Pass cells through 30 µm Pre-Separation Filter (Miltenyi Biotec, 130-041-407) into a 15mL conical tube.
- Count cells on a hemocytometer (2 replicates per sample) with Trypan blue. This will generate the final concentration to determine the loading volume onto the 10X Genomics chip.
- Proceed to cell loading for 10X Genomics 5′ HT v2 scRNA-seq assay.