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Single-cell RNA sequencing from human dorsal root ganglion V.1

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We use this protocol and it's working

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Abstract

This protocol describes how to extract single cells from dorsal root ganglia sourced from human organ donors and perform single-cell RNA sequencing using the Single Cell Gene Expression FLEX kit from 10X Genomics.

Materials

Materials Needed:

1. Wet Ice
2. Bonn scissors
3. Sterile dissection dishes (Pyrex Petri Dish 150 mm x 15 mm)
4. DNA lo bind tubes 1.5ml (0030108418)
5. 15 ml conical tubes
6. Cell Strainer 100µm (352360)
7. Hemocytometer/ cell counter
8. 50 ml conical Tube
9. RNAse Zap (ThermoFisher, AM9782)
10. 0.4% Trypan (ThermoFisher Scientific, 15-250-061)

Chemicals:

1. N-Methyl-D-glucamine - (Sigma, cat no. M2004-500G)
2. HCl - 12.1 N (Fisher, cat. no. A144-212
3. KCl - (Sigma, cat. no. P5405-250G)
4. NaH2PO4 - (Sigma, cat. no. S5011-100G)
5. NaHCO3- (Sigma, cat. no. S5761-1KG)
6. HEPES - (Sigma, cat. no. H4034-500G)
7. D-(+)-Glucose (Sigma, cat. no. G8270-1KG)
8. L-Ascorbic acid (Sigma, cat. no. A5960-25G)
9. Thiourea (Sigma, cat. no. T8656-50G)
10. Na+ pyruvate (Sigma, cat. no. P2256-25G)
11. MgSO4 (Fisher, cat. no. BP213-1)
12. CaCl2 dihydrate (Sigma, cat. no. C7902-500G)
13. N-acetylcysteine (Sigma, cat. no. A7250-50G)
14. Hank's Balanced Salt Solution (HBSS) without calcium and magnesium
15. STEMxyme (Worthington Biochemical, LS004106)
16. Bovine serum Albumin (Biopharm laboratories, 71-015-025)
17. Deoxyribonuclease I (Worthington Biochemical, LS002139)
18. Phosphate buffer Saline (PBS)

Library Prep kits:

Chromium Next GEM Single Cell Fixed RNA Sample Preparation Kit (PN-1000414)
Chromium Next GEM Chip Q Single Cell Kit, 16 rxns (PN-1000422)
Chromium Fixed RNA Kit, Human Transcriptome, 4 rxns x 16 BC (PN-1000476)

Before start

Required PPE: Ensure proper personal protective equipment (PPE), including a lab coat and gloves, is always worn. Before beginning, thoroughly clean pipettes and the lab bench with 70% ethanol, followed by an RNase decontamination solution like RNaseZap

Harvesting and isolating single cell from human dorsal root ganglia

1 Human dorsal root ganglia (DRGs) from lumbar levels L3-L5 are surgically extracted from organ donors with no known history of chronic pain at the Southwest Transplant Alliance (STA) 2-4 hours after cross-clamp.

1.1 The human dorsal root ganglion immediately placed in chilled and bubbled aCSF, containing:

- 93 mM N-Methyl-d-glucamine (NMDG),
- HCl (12 N): to adjust pH
- 2.5 mM KCl,
- 1.25 mM NaH2PO4,
- 30 mM NaHCO3,
- 20 mM HEPES,
- 25 mM d-(+)-Glucose,
- 5 mM l-Ascorbic acid,
- 2 mM Thiourea,
- 3 mM Sodium pyruvate,
- 10 mM MgSO4,
- 0.5 mM CaCl2 dihydrate,
- 12 mM N-acetylcysteine.

The solution was made to pH 7.4.

1.2 The DRGs were transported from the tissue recovery site to the University of Texas at Dallas for further isolation.

1.3 A detailed protocol on tissue recovery, preparation of aCSF, and transport can be found at **dx.doi.org/10.17504/protocols.io.kqdg32qr1v25/v1**

1.4 The extracted DRGs are transferred to a sterile petri dish on ice, where they are carefully trimmed using forceps and Bonn scissors to remove connective tissue, fat, spinal, and peripheral nerve processes. The dural layers (perineurium and epineurium) are meticulously removed, leaving only the ganglia bodies.

1.5 These ganglia are further divided into approximately 2 mm thick sections using Bonn scissors.

1.6 The tissue fragments are placed in sterile 5 mL of prewarmed enzyme mix containing Stemxyme (2mg/ml) and Deoxyribonuclease I (0.1mg/ml) in sterile filtered Hank's Balanced Salt Solution (HBSS) without calcium and magnesium.

1.7 This mixture is then subjected to enzymatic digestion in a shaking water bath at 37˚C. The DRGs are gently triturated through a sterile fire-polished glass Pasteur pipette every 25 minutes

until the solution becomes cloudy and the tissue chunks smoothly pass through the pipette without resistance. This process takes roughly three to four hours.

1.8 Following enzymatic digestion, the dissociated DRGs are passed through a 100 µm cell strainer to remove debris and obtain a uniform cell suspension.

1.9 The DRG cells are further purified by layering the cell suspension on a 3ml of 10% Bovine Serum Albumin (BSA) solution prepared in sterile HBSS.

1.10 The BSA gradient is then centrifuged at 900g for 5 minutes at room temperature, resulting in a cell pellet of DRG cells.

1.11 The supernatant was removed, and the cell pellet was washed and resuspended in 1 ml of PBS. A 10 µl aliquot of the cell suspension was mixed with an equal volume of 0.4% Trypan blue and used for counting with a hemocytometer to calculate the total number of cells accurately.

1.12 The cells were centrifuged at 850g for 5 minutes, and the cell pellet is ready for fixation.

Fixation and probe hybridization for single-cell RNA sequencing

2 The cell pellet is immediately fixed using the Chromium Next GEM Single Cell Fixed RNA Sample Preparation Kit. 300,000- 10X10^6 cells per one ml of the fixation buffer is added to the cell pellet and carefully pipetted up and down to create a homogeneous cell suspension. https://cdn.10xgenomics.com/image/upload/v1704391365/support-documents/CG000478_DemonstratedProtocol_Cell_NucleiFixation_Chromium_FixedRNA_Profiling_RevD.pdf

2.1 The whole cells are then fixed for 17 hours at 4°C.

2.2 The fixed cells are resuspended in 2 ml of phosphate buffer saline (PBS) and centrifuged at 850g for 5 minutes.

2.3 The supernatant is removed, and the cell pellet is resuspended in 1 ml of quenching buffer containing 100µl of enhancer, as provided in the 10X single-cell fixed RNA sample preparation kit (1000414)

2.4 The fixed samples can be stored for one week at 4°C (short-term storage) or six months at -80°C (long-term storage) by adding 50% glycerol for a final concentration of 10% before proceeding to the library preparation.

Library preparation for single-cell RNA sequencing

3 The cells are then incubated for 16 hours with the probe hybridization provided by 10X Genomics (Chromium Fixed RNA profiling kit, Human Transcriptome, 1000476).

3.1 NOTE: To compare other datasets, it is essential to maintain the same fixation and probe hybridization times for subsequent single-cell sequencing experiments.

3.2 The library preparation is completed according to the manufacturer's protocol. **https://cdn.10xgenomics.com/image/upload/v1722287750/support-documents/CG000527_Chromium_FixedRNAProfiling_MultiplexedSamples_UserGuide_Rev_F.pdf**)

3.3 The libraries were sequenced using Nextseq2000 at the University of Texas at Dallas Genome Core.

Processing of single-cell RNA sequencing

4 Sequencing data were processed and mapped to the human (GRCh38) genome using 10X Genomics Fix RNA profiling Cellranger multi pipeline (v7)

4.1 The processed sequencing data output from the Cellranger pipeline can be further analyzed using packages such as Seurat or Scanpy.