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Single nuclei RNA sequencing from human dorsal root ganglion

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PRECISION Human Pain ...

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We use this protocol and it's working

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Abstract

This protocol describes extracting nuclei from human dorsal root ganglia for single nucleus transcriptomics.

Materials

- Wet Ice
- Bonn scissors
- Forceps
- Sterile dissection dishes (Pyrex Petri Dish 150 mm x 15 mm)
- DNA lo bind tubes 1.5ml (Fisher Scientific ,13-698-791)
- 15 ml conical tubes
- Cell Strainer 100µm (Falcon, 352360)
- Cell Strainer 70µm (Falcon, 50828738)
- Hemocytometer/cell counter
- 50 ml conical tube
- Glass Dounce homogenizer
- Centrifuge

Chemicals:

- Dulbecco's phosphate-buffered saline (DPBS) without calcium and magnesium (ThermoFisher, 14190144)
- Triton X-100 (Sigma, T8787)
- Potassium Chloride (2M) (ThermoFisher, AM9640G)
- Magnesium Chloride (1M) (ThermoFisher, AM9530G)
- Tris Buffer pH8.0 (1M) (ThermoFisher, AM9856)
- Dithiothreitol (DTT) (ThermoFisher, D1532)
- Complete protease inhibitor (Sigma, 11836153001)
- RNAsin (Promega, N2515)
- Bovine Serum Albumin (BSA, Fisher Scientific 50-753-3073)
- Sucrose(Sigma, S1888)
- RNase Zap (Thermo Scientific, AM9782)
- 0.4% Trypan blue (ThermoFisher Scientific, 15-250-061)

Fixation and library Prep kits:

- Chromium Next GEM Single Cell Fixed RNA Sample Preparation Kit (PN-1000414)
- Chromium Fixed RNA Profiling FLEX (Human Transcriptome, Catalog #1000476)

Before start

Necessary PPE: Always wear appropriate personal protective equipment (PPE), including a lab coat and gloves. Before beginning, thoroughly clean pipettes and the lab bench with 70% ethanol, followed by an RNase decontamination solution like RNaseZap for extra protection.

- Gather ice.
- Prepare buffers.
- Set the centrifuge to 4°C.
- Chill the Dounce homogenizer and spray it with RNase Away.

Solution preparation

50ml of the **nuclei isolation buffer (NIB)** is made by adding concentrations of
- 0.25M sucrose
- 150mM KCl
- 5mM MgCl2
- 1M Tris Buffer pH 8.0
- Water to make the solution

The solution was made to pH 8.0.

*This solution can be stored at 4°C for up to a month.

On the day of, 50ml **fresh homogenization buffer** is made by adding
- 48.25ml of the above stock (NIB)
- 0.1mM DTT
- complete protease inhibitor (1 tablet)
- 0.1% Triton-X 100
- 0.2U/µl RNAsin

Wash/resuspension buffer prepared on the day of by adding
- 1% BSA (BSA should be RNAse free)
- 0.2U/µL RNase Inhibitor
- 1X PBS (Use PBS without calcium and Magnesium to prevent clumping of cells)

Nuclei Isolation from human dorsal root ganglion

1 DRG tissues from organ donor or surgical samples were harvested as previously described in dx.doi.org/10.17504/protocols.io.kqdg32qr1v25/v1, dx.doi.org/10.17504/protocols.io.e6nvw1×6wlmk/v1, dx.doi.org/10.17504/protocols.io.eq2lywoervx9/v1

2 The snap-frozen DRG tissue is transferred to a sterile petri dish on ice, and the dural layers and connective tissues are carefully trimmed away using forceps and Bonn scissors, leaving only the ganglia bodies.

2.1 The ganglion is chopped into small pieces (less than 1mm) using scissors in a 1.5 mL RNase-free tube with 500µl of homogenization buffer.

2.2 The tissue was then transferred to a glass Dounce homogenizer containing chilled homogenization buffer, and homogenized with approximately 15 strokes of the pestle.

2.3 The homogenate was filtered using a 70µm strainer mesh (for larger cells like human DRG neurons, a 100µm strainer is used; for smaller cells like mouse DRG, a 40µm strainer is recommended) in a 50ml falcon tube.

2.4 The filtered homogenate was then centrifuged at 500 x g for 5 minutes at 4°C.

2.5 The supernatant was removed, and the nuclei were resuspended in 500µL of wash/resuspension buffer and centrifuged again at 500 x g for 5 minutes at 4°C.

2.6 Before counting the nuclei suspension is filtered through a 70µm strainer mesh to obtain a single-cell suspension and remove excess myelin.

2.7 A 10 µL aliquot of the nuclei suspension is mixed with an equal volume of 0.4% Trypan blue and counted using a hemocytometer to accurately determine the total number of nuclei. The optimal nuclei concentration for successful library preparation is approximately 1000 nuclei/µL.

2.8 Inspect nuclear membrane integrity under a microscope at **40x or 60x magnification** to verify the absence of blebbing. Use the following criteria to assess nuclei quality:

- **Type A:** High-quality nuclei with distinct, smooth edges. Optimal for single-cell gene expression library preparation.
- **Type B:** Mostly intact nuclei with minor blebbing. Acceptable for library preparation, but use caution.

- **Type C:** Nuclei show significant blebbing. Proceed only if necessary, acknowledging increased risk to library quality.
- **Type D:** Nuclei severely damaged or ruptured. **Do not proceed** with library preparation. (https://kb.10xgenomics.com/hc/en-us/articles/360050780051-What-are-the-best-practices-for-working-with-nuclei-samples-for-3-single-cell-gene-expression)

2.9 If the nuclei quality is good, proceed by centrifuging the suspension at 850 × g for 5 minutes.

Fixation of nuclei for profiling

3
- The nuclei pellet is immediately fixed using the Chromium Next GEM Single Cell Fixed RNA Sample Preparation Kit (PN-1000414). 3×10^5-10×10^6 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

3.1 Fix nuclei for approximately 16-17 hours at 4°C.

3.2 After fixation, resuspend the nuclei gently in 2 mL phosphate-buffered saline (PBS). Centrifuge the suspension at 850g for 5 minutes.

3.3 Carefully discard the supernatant. Resuspend the resulting nuclei pellet in 1 mL of freshly prepared quenching buffer supplemented with 100 µL of enhancer (provided in the 10X single-cell fixed RNA preparation kit, catalog number 1000414).

3.4 For short-term storage (up to 1 week), keep the fixed nuclei at 4°C. For long-term storage (up to 6 months), add glycerol to a final concentration of 10% (v/v), and store at -80°C. Proceed to library preparation after storage.

Library Preparation for Single-nuclei RNA Sequencing

4 Next, follow the 10x Genomics Chromium Fixed RNA Profiling FLEX kit protocol for preparing the probe hybridization solution (Human Transcriptome, Catalog #1000476), and incubate for approximately 16-17 hours.

4.1 **Important:** Maintain consistent fixation and probe hybridization times across experiments if planning to compare results with additional datasets.

4.2 Complete the library preparation following the manufacturer's instructions provided by 10X Genomics (detailed protocol https://cdn.10xgenomics.com/image/upload/v1722287750/support-documents/CG000527_Chromium_FixedRNAProfiling_MultiplexedSamples_UserGuide_Rev_F.pdf).

4.3 Sequence prepared libraries on a NextSeq2000 sequencer (or equivalent), as performed at the University of Texas at Dallas Genome Core Facility.

Data Processing of Single-nuclei RNA Sequencing

5 Map sequencing data to the human genome (reference: GRCh38) using the Cell Ranger multi pipeline (version 7) from 10X Genomics.

5.1 Perform downstream analyses of the Cell Ranger output data using computational tools such as Seurat (R package) or Scanpy (Python package).