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10X Genomics Single-Nucleus RNA-Sequencing for Transcriptomic Profiling of Adult Human Tissues V.1

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[1]UCSD

KPMP | Human BioMolecular Atl...

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We use this protocol and it's working

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Abstract

10X Genomics Single Cell 3' (v3) RNA sequencing is a microdroplet-based method that permits the effective capture and sequencing of the mRNA and pre-mRNA molecules from single nuclei [1]. RNA molecules are transcribed and processed within the nucleus before exporting to ER for translation into proteins. As such, nuclear RNA is a mixture of nascent transcripts, partially or fully processed mRNA, and various non-coding RNA molecules. The total RNA content within the nucleus is roughly 10% of the RNA content in a whole cell, but has been found to accurately represent whole cell expression values in adult human tissues [2,3] including the kidney [4]. Nuclei can be readily isolated from frozen tissues with a combination of chemical and physical treatments that can effectively circumvent the non-uniform or incomplete dissociation of solid tissues into single cells, as well as RNA degradation or artefacts (such as stress response) during dissociation. Here we present a modified version of the published 10X protocol [1] that we have adapted for the processing of adult human kidney nuclei.

References

1. Chromium Single Cell 3' Reagent Kits v3 User Guide (Rev A) CG000183, support.10xgenomics.com.
2. Lake et al. (2016). Science, doi:10.1126/science.aaf1204.
3. Lake et al. (2018). Nature Biotechnology, doi:10.1038/nbt.4038.
4. Lake et al. (2019). Nature Communications, doi:10.1038/s41467-019-10861-2.

Guidelines

Full protocol is from 10X Genomics. All modifications are to the original protocol (Chromium Single Cell 3' Reagent Kits v3 User Guide (Rev A) CG000183, support.10xgenomics.com)

Materials

MATERIALS

Chromium™ Single Cell 3 GEM Library & Gel Bead Kit v3 **10x Genomics Catalog #**1000075

Chromium Chip B Single Cell Kit **10x Genomics Catalog #**1000074

Chromium i7 Multiplex Kit **10x Genomics Catalog #**120262

Nuclease-free water
Ethanol (200 proof)
Dynabeads MyOne Silane
SPRI select reagent set
50% glycerol
10% Tween 20
Low TE Buffer (10mM Tris-HCl, pH 8.0, 0.1 EDTA)
Qiagen buffer EB
Qubit dsDNA HS Assay Kit
PCR strip tubes with flat cap
LoBind 1.5 ml tubes
Qubit Assay tubes

Troubleshooting

Nuclei isolation protocol [1] modified for kidney tissue

1 Prepare NEB containing 1:1000 dilution of DAPI and 0.1% RNAse Inhibitor (RI), chill on ice

2 Treat dounce with RNAseZap, rinse with sterile water (if possible: UV treat 00:15:00)

3 Transfer vial containing tissue (e.g. 7×40 µm cryosections for ~5 mm2 total, ranging 1-20 mm3; from above) to ice

4 For sections in RNAlater, wash briefly with PBSE and immediately proceed to substep below

4.1 Determine stock nuclei concentration using and appropriate cell counter

4.2 Dilute nuclei stock to bin in the 700-1200 nuclei per uL range in 50 µL total volume using the dilution guide

Note

If nuclei concentrations fall below target range, then centrifugation can be performed to increase nuclei concentrations to be within range
- Start with nuclei stock in PBS with 0.1% BSA
- Spin 900xg for 10 minutes
- Resusupend in appropriate volume PBS
- Use 10 uL to count; 50 uL for loading
-

QC cutoff: minimum of 10,000 nuclei (for 200 nuclei per uL in 50 uL total volume)

Safety information

Caution: BSA is necessary to avoid clumping and prevent nuclei loss from sticking to the tube during the spin down. DO NOT USE more than .01% BSA in nuclei stock

Isolate Nuclei

5 Add 1 mL ice cold NEB/DAPI/RI buffer to tissue segments using p1000 and pipette up and down ~10 times, transfer to dounce homogenizer

6 Cut the end off of a p1000 tip to increase bore size using a sterile scalpel, then pipette sections up and down to disperse and dissolve OCT, ~20 times

7 Using regular p1000 tip, pipette ~10x to further dissociate tissues into manageable sizes (note: tissue needs to be able to pass through a p1000 tip easily before proceeding), transfer to dounce homogenizer

8 Gently dounce tissue on ice:
- 5 strokes with pestle A
- ~20 strokes with pestle B (minimize bubble formation)

9 Transfer solution to a 15 mL tube.

10 Wash dounce with 1 mL NEB/DAPI/RI buffer and add this into the same tube

11 Incubate on ice 00:10:00

12 Pass supernatant through 30-µm CellTrics filter to a new 15 mL conical tube

13 Bring up to 10 mL with PBSE

14 Pellet nuclei: 900 g , 00:10:00 at 4 °C

15 To use nuclei directly for snDrop-seq, proceed to 2.3.4.

16 To use nuclei on a later date for snDrop-seq:
- Remove supernatant and resuspend pellet in 100 µL PBSE+1%BSA
- Add 900 µL RNAlater, incubate at 4 °C 1-2 hours then store at -20 °C up to 1 month
- To remove RNAlater, centrifuge nuclei at 4000g, 00:10:00 at 4 °C. Proceed to method (e.g. 2.3.4)

17 Count nuclei (e.g. BioRad T20 Cell Counter)

18 Check nuclei integrity under fluorescent microscope using DAPI channel. Nuclei should appear distinct, have rounded borders and the majority occurring as singlets. High clumping rates would indicate damaged nuclei and would require re-filtering using 30-µm CellTrics filter or exclusion. At least 10,000 nuclei are needed to proceed with 10X 3′ RNA v3.

GEM Generation and Barcoding

19 Prepare Reagents for use

1. Equilibrate gel beads to room temperature for 00:30:00 .
2. Thaw, vortex, and centrifuge RT Reagent, Template Switch Oligo, and Reducing Agent B. Verify no precipitate in Reducing Agent B.
3. Centrifuge RT Enzyme C before adding to Master Mix.

20 Prepare Master Mix

1. Prepare on ice. Pipette mix 15x and centrifuge briefly.
2. Add 33.4 µL Master Mix to each tube of a PCR 8-tube strip on ice.

21 Load Chromium Single Cell B Chip

1. Assemble Chromium Chip B in a 10X Chip Holder.
2. Must load chip in order according to row label for microfluidic channels to work properly: Row 1 → Row 2 → Row 3
3. Make sure NO bubbles are introduced while loading chip.
4. Dispense 50% glycerol solution into unused Chip Wells (if <8 samples used per chip)
   a. 75 µL into unused wells in Row 1
   b. 40 µL into unused wells in Row 2
   c. 280 µL into unused wells in Row 3
5. Use Cell Suspension Volume Calculator Table to add the appropriate volume of nuclease-free water and corresponding volume of nuclei stock to make working nuclei suspension
6. Nuclei Loading
   a. Minimum: load 800 nuclei → target recovery 500 nuclei

b. Maximum: load 1600 nuclei → target recovery 10000 nuclei

7. Add 46.6 µL working Nuclei Suspension to the Master Mix in PCR tubes on ice for 80 µL total volume
8. Load 75 µL Master Mix and Nuclei Suspension into the bottom center of each well in Row 1 without introducing bubbles.
9. Snap the Gel Bead strip into a 10X Vortex Adapter. Vortex 00:00:30 . Remove the Gel Bead strip and flick sharply downward to ensure maximum recovery. Confirm there are no bubbles at the bottom of the tubes and liquid levels look even.
10. Load 40 µL gel beads in Row 2. Only puncture the foil seal for gel bead tubes being used. Dispense slowly and without introducing bubbles.
11. Load 280 µL partitioning oil into each Row 3 by pipetting two aliquots of 140 µL .
12. Attach 10X gasket. Align the top-notch. Ensure gasket holes are aligned with the wells. Avoid touching the smooth gasket surface. Do not press down on the gasket.

22 Run the Chromium Controller

1. Press the eject button on the controller to eject.
2. Place the assembled chip with the gasket in the tray. Press the button to retract the tray.
3. Confirm the program on screen. Press the play button.
4. At completion of the run (~ 00:07:00), the controller will chime. Proceed immediately to the next step.

23 Transfer GEMs

1. Chill strip tubes on ice.
2. Press the eject button to remove the chip.
3. Discard the gasket. Open the chip holder. Fold the lid back until it clicks to expose the wells at 45 degrees.
4. Check the volume in rows 1-3. Abnormally high volume in any well indicates a clog.
5. Slowly aspirate 100 uL GEMs from the lowest points of the Recovery Wells without creating a seal between the pipette tips and the wells.

Note

GEMs should appear opaque and uniform across all channels. Excess partitioning oil (clear) in the pipette tips indicates a potential clog.

6. Slowly dispense (~ 00:00:20) GEMs into the tube strip on ice with the pipette tips against the sidewalls of the wells.

24 GEM-RT Incubation

1. Incubate in a thermocycler to complete reverse transcription.
2. Store at 4 °C for up to 72:00:00 or at -20 °C for up to a week. Or proceed to the next step.

25 Post GEM-RT Cleanup & cDNA Amplification - Prepare Reagents for use

1. Thaw, vortex, and centrifuge a tube of Reducing Agent B and cDNA primers.
2. Maintain Amp Mix on ice after vortex and centrifuge
3. Thaw Cleanup Buffer at 65 °C for 00:10:00 with shaking at max rpm then cool to room temperature.

26 Post GEM RT-Cleanup --Dynabead

1. Add 125 µL Recovery Agent to each sample at room temperature. DO NOT MIX. Wait 00:01:00 .
2. Carefully aspirate 125 µL of pink oil phase from the bottom of the tube. DO NOT aspirate any aqueous sample.
3. Prepare Dynabeads Cleanup Mix
4. Vortex and centrifuge mixture and add 200 µL to each sample. Pipette 5x to mix.
5. Incubate at room temperature for 00:10:00 ; pipette mix again 00:05:00 into incubation.
6. Prepare Elution Solution I. Vortex and centrifuge briefly.
7. Place sample on magnetic separator and remove the supernatant.
8. Wash twice with freshly prepared 80% ethanol.
9. Elute with 35.5 uL Elution Solution I.
10. Transfer 35 uL to a new strip tube.

27 cDNA Amplification

1. Prepare cDNA Amplification Mix on ice. Vortex and centrifuge briefly.
2. Add 65 µL cDNA Amplification Reaction Mix to 35 µL sample.
3. Pipette mix 15x to mix and centrifuge briefly.
4. Incubate in a thermocycler to amplify cDNA.

Note

- Use cycle number optimization table for total number of cycles
- When using nuclei, increase the optimized number of cycles by one cycle
- Maximum: load 1600 nuclei - perform 12 cycles

28 cDNA Cleanup --SPRIselect

1. Perform a 0.6X beads purification with SPRIselect reagent
2. Wash twice with freshly prepared 80% ethanol
3. Elute with 40.5 µL Qiagen Buffer EB.
4. Transfer 40 µL to a new strip tube.
5. Store at 4 °C for up to 72:00:00 or at -20 °C for up to 4 weeks. Or proceed to the next step.

29 cDNA QC & Quantification

1. Run TapeStation to obtain concentration and size
2. Expected size range for amplified cDNA: 400 to 2500 bp
3. cDNA total yield range: 80 to 1000 ng

Note

QC Cutoff: Minimum cDNA total yield of 80 ng

3' Gene Expression Library Construction

30 Prepare Reagents for use

1. Thaw, vortex, and centrifuge Fragmentation Buffer, Adaptor Oligos, Ligation Buffer, SI Primer.
2. Maintain on ice Fragmentation Enzyme, DNA Ligase, and Amp Mix.

31 Fragmentation, End Repair & A-tailing

1. Prepare Fragmentation Mix on ice. Pipette mix and centrifuge briefly.
2. Transfer 10 µL purified cDNA to a new strip tube

Note

Note that 10 uL (25%) cDNA sample is sufficient for generating 3' Gene Expression Library. The remaining 30 uL (75%) cDNA sample can be stored at 4 C for up to 72 hours or at -20 C for up to 4 weeks for generating additional 3' Gene Expression Libraries.

3. Add 25 µL Buffer EB to each sample
4. Add 15 µL Fragmentation Mix to each sample
5. Pipette mix, centrifuge briefly, and transfer to pre-cooled block. “SKIP” hold step to initiate the protocol

32 Post Fragmentation, End Repair & A-tailing Double Sided Size Selection --SPRIselect

1. Vortex to resuspend the SPRIselect reagent. Add 30 µL SPRIselect reagent (0.6X) to each sample and pipette mix 15x.
2. Incubate 00:05:00 at room temperature.
3. Centrifuge briefly
4. Place sample on magnetic separator. DO NOT discard supernatant.
5. Transfer 75 µL supernatant to a new tube strip tube.
6. Vortex to resuspend the SPRIselect reagent. Add 10 µL SPRIselect reagent (0.8X) to each sample and pipette mix 15x.
7. Place sample on magnetic separator and remove supernatant. DO NOT discard any beads.
8. Wash twice with freshly prepared 80% ethanol
9. Elute with 50.5 µL Qiagen Buffer EB.
10. Transfer 50 µL to a new strip tube.

33 Adaptor Ligation

1. Prepare Adaptor Ligation Mix on ice. Pipette mix and centrifuge briefly.
2. Add 50 µL Adaptor Ligation Mix to sample. Pipette mix 15x. Centrifuge briefly.
3. Incubate in a thermocycler to ligate adaptor.

34 Post Ligation Cleanup --SPRIselect

1. Perform a 0.8X beads purification with SPRIselect reagent

2. Wash twice with freshly prepared 80% ethanol
3. Elute with 30.5 µL Qiagen Buffer EB.
4. Transfer 30 µL to a new strip tube.

35 Sample Index PCR

1. Choose the appropriate sample index sets to ensure that no sample indices overlap in a multiplexed sequencing run.
2. Prepare Sample Index PCR Mix on ice. Pipette mix and centrifuge briefly.
3. Add 60 µL Sample Index PCR Mix to the sample
4. Add 10 µL of individual Chromium i7 Sample Index (from SI-GA plate) to each well. Record well assignment. Mix and centrifuge briefly.
5. Incubate in a thermocycler to incorporate sample indices.

> Note
>
> Use cycle number recommendation table for total number of cycles; recommended number of cycles is based on cDNA input

6. Store at 4 °C for up to 72:00:00 . Or proceed to the next step.

36 Post Sample Index PCR Double Sided Size Selection --SPRIselect

1. Vortex to resuspend the SPRIselect reagent. Add 60 µL SPRIselect reagent (0.6X) to each sample and pipette mix 15x.
2. Incubate 00:05:00 at room temperature.
3. Centrifuge briefly
4. Place sample on magnetic separator. DO NOT discard supernatant.
5. Transfer 150 µL supernatant to a new tube strip tube.
6. Vortex to resuspend the SPRIselect reagent. Add 20 µL SPRIselect reagent (0.8X) to each sample and pipette mix 15x.
7. Incubate 00:05:00 at room temperature.
8. Centrifuge briefly
9. Place sample on magnetic separator and remove supernatant. DO NOT discard any beads.
10. Wash twice with freshly prepared 80% ethanol xi. Elute with 35.5 µL Qiagen Buffer EB.
11. Transfer 35 µL to a new strip tube.
12. Store at 4 °C for up to 72:00:00 or at -20 °C for long-term storage.

Note

QC Post Library Construction:
- **Quantify Library (e.g. using Qubit dsDNA HS Assay)**
- **Estimate Library size range (e.g. using TapeStation or BioAnalyzer) - exprected size range of 300 to 800 bp, average: 475 bp**

Sequencing

37 MiSeq Sequencing - **QC for estimation of library quality and number of nuclei captured**

1. Paired End, Single Indexing
    a. Read 1: 28 cycles
    b. i7 Index: 8 cycles
    c. i5 Index: 0 cycles
    d. Read 2: 91 cycles
2. Library Loading
    a. 10X recommended Loading concentration: 11 pM
    b. Optional: 1% PhiX
3. Output
    a. 22-25 million reads

38 NovaSeq Sequencing (target - 25,000-50,000 reads per nucleus)

1. Paired End, Single Indexing
    a. Read 1: 28 cycles
    b. i7 Index: 8 cycles
    c. i5 Index: 0 cycles
    d. Read 2: 91 cycles
2. Library Loading
    a. 10X recommended Loading concentration: 300 pM
    b. Optional: 1% PhiX
3. Output

a. SP: 650–800 million reads
b. S1: 1.3–1.6 billion reads
c. S2: 3.3 –4.1 billion reads
d. S4: 8-10 billion reads

Cell Ranger Mapping and Analysis Pipeline

39 Generate pre-mRNA reference

Note

IMPORTANT: for nuclei experiments, we need to use the reference files with pre-mrna in order to count intronic reads in the UMI counts

```
awk 'BEGIN{FS="\t"; OFS="\t"} $3 == "transcript"{ $3="exon"; print}' \
    refdata-cellranger-GRCh38-1.2.0/genes/genes.gtf > GRCh38-1.2.0.premrna.gtf

cellranger mkref --genome=GRCh38-1.2.0_premrna \
                 --fasta=refdata-cellranger-GRCh38-1.2.0/fasta/genome.fa \
                 --genes=GRCh38-1.2.0.premrna.gtf
```

b. Generate Sample Sheet

Note

Use sample sheet generator provided by 10X Genomics to generate a "SampleSheet.csv":**Sample Sheet Generator**

c. Generate fastq files

Note

Use **mkfastq** command

```
cellranger mkfastq --use-bases-mask y28,i8,y91 --sample-sheet SampleSheet.csv -R
/path/to/seq-files/190625_M00159_0214_000000000-CH9WH/ --ignore-dual-index
```

d. Run count for each sample

```
cellranger count --id SAMPLE01 \
--transcriptome=/path/to/genome/GRCh38-3.0.0_premrna \
--fastqs=CH9WH \
--sample=SAMPLE01 \
--expect-cells=5000 \
--chemistry=threeprime
```