Single cell RNA sequencing (scRNA-seq)

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1 Works for me

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ABSTRACT

Previously, RNA sequencing for whole-genome gene expression analysis could only be performed on whole tissue (bulk RNA seq), or microdissected tissue compartments, where gene expression measurements reflect an average across all captured cell types. Single cell RNA sequencing allows the measurement and comparison of gene expression of individual cells and thus capture the previously underappreciated cellular heterogeneity within tissues such as the kidney. Our protocol allows the dissociation of a single kidney biopsy core to a single cell suspension. The total mRNA from each living cell is then captured, uniquely barcoded, and gene expression profiled with RNAseq. Single cell transcriptomics of adult kidney tissue is performed using 10X Genomics droplet-based technology. 10X Genomics technology combines microfluidics with molecular barcoding and custom bioinformatics software (CellRanger). High-throughput single cell transcriptomic measurements enable profiling of individual cell types.

GUIDELINES

After tissue dissociation into single cell suspension all further steps are performed on ice (4ºC).

GEM Generation and Barcoding

1.Notes on Workflow
a)Operating temperature: 64 – 82 ºF (18 – 28 ºC)
b)Timely preparation and proper loading of reagents is crucial
c)Use recommended plastics when handling GEMs
·Some plastics can interfere with emulsion stability
d)Minimize exposure of opened reagents, chips, and gaskets to general lab environment
·Microfluidic systems can clog due to the introduction of particulates
·If available, chip preparation and loading can be completed in a laminar flow PCR hood

MATERIALS

NAME	CATALOG #	VENDOR
Centrifuges 5810 R	View	Eppendorf Centrifuge
1.5 mL Eppendorf tubes		
Glass pasteur pipettes		
15 ml conical tubes		
Cryovial, PP, 1.8ml, external cap, 13mmx46mm, sterile,with Silicone washer	SP54223.SIZE.1CS	Bio Basic Inc.
Thermomixer C or R	5382000015 / Z605271	Eppendorf
HypoThermosol® FRS 100 mL	7935	Stemcell Technologies
CryoStor® CS10 100 mL	7930	Stemcell Technologies
Fetal bovine serum		

NAME	CATALOG #	VENDOR
Liberase TL	05 401 020 001	Roche
DMEM F12/HEPES	113300	Gibco - Thermo Fischer
Pre-Separation Filters (30 µm)	130-041-407	Miltenyi Biotec
Nunc™ IVF Petri Dishes, Untreated; 35 x 10mm petri dish	150255	Thermo Fisher

BEFORE STARTING

Prepare

- "Stop" solution = RPMI + 10% FCS

Make sure equipment at right temp

- 37°C shaker
- 4°C centrifuge

Freezing in Cryostor

1 Remove biopsy frozen in **500 µl** CryoStor solution in a **1.8 ml** cryovial out of the liquid nitrogen tank

. Thaw frozen biopsy in a **37 °C** water bath under constant swirling for 60-90 sec only until biopsy at the bottom is ice-free.

2 Place the **1.8 ml** cryovial with **1.5 ml** HypoThermosol on ice

3 Add the renal biopsy specimen to the cryovial with HypoThermosol immediately.

4 Keep upright and at **4 °C** (on ice) between the collection step and the cryopreservation step.

5 Place the **1.8 ml** cryovial with **1.5 ml** CryoStor on ice.

6 Transfer the renal biopsy specimen to the cryovial with CryoStor CS10 very quickly.

7 Keep the cryovial upright and on ice for **00:15:00** to allow permeabilization.

8 Place the cryovial in a Mr. Frosty freezing unit containing isopropanol.

9 Place the Mr. Frosty container in the **-80 °C** freezer for **24:00:00** .

10 Transfer the cryovial from the Mr. Frosty container to liquid N2, submerged. (Cryovials can be shipped on dry ice)

Thawing and Cell Dissociation

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20 Transfer biopsy to plastic petri dish (ø 3.5 cm) filled with 1 ml DMEM/F12/10%FBS for 00:00:10 to wash off remaining DMSO at room temperature.

21 Transfer to second plastic petri dish (ø 3.5 cm) filled with 1 ml DMEM/F12/10%FBS and incubate for 00:10:00 at room temperature.

22 Take picture of biopsy together with a ruler.

23 Cut biopsy in ~1 mm long pieces using a scalpel.

24 Prepare dissociation medium: 450 µl DMEM/F12 (no FBS) and 50 µl µl Liberase TL (conc.: 2.5 mg/ml H_2O, final enzyme conc.: 0.25 mg/ml in 1.5 ml Eppendorf tube (noBind).

25 Transfer biopsy tissue to prepared dissociation medium prewarmed to **37 °C** .

26 Incubate at **37 °C** for **00:12:00** and shake at 500 rpm in an

Eppendorf Thermomixer.

27 Triturate after **00:06:00** for 15 times with wide bore **1 ml** pipette tip.

28 Stop reaction with **500 µl** DMEM/F12/10%FBS (room temperature) and gently mix.

29 Incubate for **00:01:00** at room temperature.

30 Filter through

30 µm gaze (Miltenyi) into **15 ml** tube on ice.

31 Push tissue through gaze using a **5 ml** syringe plunger (rubber side).

32 Wash filter with **10 ml** cold DMEM/F12/10%FBS.

33 Filter single cell suspension again through new 30 µm gaze (Miltenyi) into a second **15 ml** tube.

34 Wash first **15 ml** tube with **1 ml** cold DMEM/F12/10% FBS and apply through 30 µm gaze into second **15 ml** tube.

35 Centrifuge cell suspension at 200 x *g* for **00:10:00** at **4 °C** . 1m

36 Remove supernatant using glass Pasteur pipette and house vacuum. (~ **20 µl** remain)

37 Add and dispense cell pellet carefully with **40 µl** DMEM/F12/10%FBS.

38 Take **5 µl** for cell count and mix with **5 µl** Trypan blue.

39 Count viable cells on hemocytometer or with automated cell counter (Countess)

40 Submit to 10X Genomics Chromium platform.

41 Load up to 10,000 viable cells in up to **46 µl** of the cell suspension to 10X Chromium array ASAP.

Cell Handling Recommendations

42 A fully dissociated, single-cell suspension is essential for the analysis of single-cell transcriptomes. a) Suspension cell lines, bead-enriched and flow-sorted cells can be used directly after washing. b) Adherent cell lines require trypsin treatment first. c) Single-cell suspension from tissues requires optimization of dissociation.

43 It is important to treat cells gently to minimize cell lysis and loss: a) When cells lyse, the mRNA will contaminate other GEMs. b) Wash and resuspend in PBS + 0.04% non-acetylated BSA to minimize cell loss during handling. c) Wash cells 2x using a wide-bore pipette tip to remove ambient RNA and contaminants.

44 Use a cell strainer to remove clumps or debris from washed cells. a) Cell debris and fibers can be counted as either dead or live cells, causing inaccurate counts. b) Cell debris and large clumps may clog or wet the chip.

45 Quantitate cells accurately before loading the system. a) Approximately 65% loaded cells will be recovered. b) Recommended range: 500 to 10,000 recovered cells. c) Under- or over-loading may impact application performance.

46 Store cells suspension on ice until you are ready to load the chip. a) Prepare cell suspension right before you are going to use it. b) Load cells as soon as possible after preparation, ideally within 30 min

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Washing Isolated Cells

52 Transfer cells in media to a **2 ml** Eppendorf tube

53 Spin down cells to form pellet • Depending on cell size and concentration, pellet size varies

54 Remove supernatant

55 Gently add 1x PBS + 0.04% BSA away from cell pellet

56 Gently pipette mix with Wide Bore pipette tip

57 Repeat the wash one more time

58 Spin down cells to form pellet

59 Remove supernatant

60 Resuspend cells in 1x PBS + 0.04% BSA with gentle pipette mix • For accurate cell counting, do not invert tubes

61 Adjust to desired cell concentration as indicated in section 3.

Preparing Single Cell Master Mix

62 Equilibrate Single Cell 3’ Gel Beads to room temperature for at least 00:30:00 , thaw reagents

63 Resuspend the RT primer in 40 µl l low TE buffer and prepare the thermal cycler.

64 Prepare Master Mix on ice. Do NOT add cells yet.

Loading the Single Cell 3' Chip

65 Place the Chip in the Holder before loading reagents

66 Handle the chip by its edges, taking care to avoid contacting the bottom surface (this helps to mitigate frictional charging)

67 Align the beveled corners at upper left and insert chip under the guide at the left edge of the recess in the holder

68 Press down on the right side of the chip until the spring loaded clip engages

69 Close the Holder and lay flat on benchtop

Loading Single Cell Master Mix

70 Dispense the **66.2 µl** of Master Mix into each well of an 8-tube strip on a chilled metal block resting on ice. Then add the calculated volume of water into each strip tube.

71 The next step is critical to recovering the maximum number of cells. It is important to ensure that: a) all of the cells in the sample are suspended b) the sample is homogenous when adding the cell suspension volume to the Master Mix.

72 Estimate the volume of the cell suspension and set the pipette at half that volume for pipette mixing. Wide bore pipette tips are not necessary for this step

73 Pipette the required volume of cell suspension from the center of the suspension volume.

74 Add the appropriate volume (µl) of single cell suspension to each well of the tube strip containing the Master Mix.

75 With a pipette set to **90 µl** gently pipette mix the combined cells and Master Mix 5 times while keeping the tube strip on a chilled metal block resting on ice.

76 Without discarding the pipette tips, transfer **90 µl** Master Mix containing cells to the wells in the row labeled 1, taking care not to introduce bubbles.

Chip Priming

77 After loading Master Mix, wait for **00:00:30**

78 This wait time is required for correct priming of channels inside the Single Cell 3’ Chip

79 Then, immediately proceed to loading the Gel Beads

Preparing Single Cell 3' Gel Beads

80 Snap the Single Cell 3’ Gel Bead Strip into a 10x Vortex Adapter and vortex for **00:00:30** . a) A 30 sec wait while vortexing the Single Cell 3’ Gel Bead Strip is required to ensure proper priming of the Master Mix containing cells in the Single Cell 3’ Chip.

81 Remove the Single Cell 3’ Gel Bead Strip and flick in a sharp, downward motion to ensure maximum recovery. Confirm that there are no bubbles at the bottom of the tube and that liquid levels are uniform.

Loading Gel Beads and Partitioning Oil

82 Pipette 40 µl l Gel Beads from Gel Bead Strip

83 Dispense Gel Beads in row labeled 2 a) Dispense into bottom of well b) Slowly pipette Gel Beads c) Avoid air bubbles

84 Transfer Partitioning Oil to a reservoir and load row labeled 3 a) Once oil is added, run the chip within 00:02:00 . The less time the chip sits, the better. b) NEVER forget to add full volume of oil – doing so can damage the Chromium™ Controller! c) Don’t leave oil in reagent reservoir for too long – avoid evaporation and contamination by particulates

85 Attach the 10x™ Gasket. The notched cut should be at the top left corner. a) Ensure the gasket holes are aligned with the chip wells. b) Do not touch or “smooth” the top surface of the gasket. c) Keep the assembly horizontal to avoid wetting the 10x Gasket with Partitioning Oil.

Running the Chromium™ Controller and transferring GEMs from the Chip

86 Load assembled Chip, Holder and Gasket.

87 Keep the Chip Holder horizontal during transfer to the instrument to avoid wetting the gasket with reagents.

88 Confirm Chromium Single Cell A program and press play button to start run.

89 The Controller will chime at the completion of the run (~ 00:06:30).

90 Maintain an Eppendorf twin.tec® 96-Well PCR plate for GEM transfer on a chilled metal block resting on ice.

91 Remove the Single Cell 3’ Chip from the Chromium Controller.

92 Open the 10x Chip Holder and fold the lid back until it clicks to expose the wells at a 45-degree angle.

93 Check for volume uniformity in the: a) Sample (1) b) Gel Bead (2) and c) Partitioning Oil (3) wells remaining in the Single Cell 3’ Chip.

94 Remove Gasket

95 Fold and lock Chip Holder at 45° angle

96 Transfer **100 µl** GEMs to twinc.tec® PCR plate a) Aspirate GEMs • Within **00:02:00** of completed run • Pipette tip just above lowest point in recovery well • Do not take more than 100 µl or double-dip b) Dispense into PCR Plate • Pipette tip against side of well • Dispense slowly (20 sec) to avoid air bubbles • Keep plate on chilled block

97 Withdraw pipette tips from the wells and verify that there is no air in the tips. GEMs should appear opaque and uniform across all channels.

GEM-RT Incubation

98 Seal plate with Bio-Rad PX1TM Plate Sealer a) Red stripe facing up b) A tight seal is critical to GEM stability

99 Cycle in Bio-Rad C1000 TouchTM thermal cycler with Deep Well Module

100 Store in the PCR plate at **4 °C** for up to **72:00:00** before proceeding to Post GEM-RT Cleanup.

Post GEM-RT Clean up

101 Add **125 µl** of Recovery Agent directly to samples in PCR plate.

102 Wait **00:01:00** .

103 With the same tips, move entire volume to 8 tube strips. (If PCR strip tubes have been used for GEM-RT reaction, no volume transfer into a new 8 tube strip is necessary) a) Make sure to extract everything from the 96 well plate b) Do not vortex or pipette mix after adding Recovery Agent

104 Slowly remove **125 µl** Recovery Agent/Partitioning Oil (pink) from the bottom of the tubes and discard a) Some Recovery Agent may remain b) Be careful to not aspirate any aqueous phase

cDNA Amplification Reaction

105 Set up cDNA Amplification Master Mix a) The optimal number of cycles for the cDNA amplification reaction is a trade-off between generating sufficient mass and minimizing PCR amplification artifacts

106 SPRI Cleanup

107 Bioanalyzer QC. Optimal performance has been characterized on input cDNA with a mean length > 2 kb.

108 Fragmentation, End Repair & A-tailing

109 Double-Sided Size Selection – SPRIselect

110 Adaptor Ligation

111 Post Ligation Cleanup – SPRIselect

112 Sample Index PCR

113 Double-Sided Size selection – SPRIselect

114 Post Library Construction QC

Library Preparation

115 Fragmentation, End Repair & A-tailing

116 Double Sided Size Selection – SPRIselect

117 Adaptor Ligation

118 Post Ligation Cleanup – SPRIselect

119 Sample Index PCR

120 Double Sided Size selection – SPRIselect

121 Post Library Construction QC

scRNA-seq sequencing and data processing

122 The library mRNA prep is sequenced on an Illumina HiSeq 4000 platform. The output from the sequencer is then analyzed using the 10XGenomics software CellRanger. This step includes the alignment of the reads to human genome (GRCH38). The raw file output from CellRanger is further processed using Seurat R package. The downstream processing includes: Log transformation, filtering out cells with < 500 genes (or < 400 UMI) and > 5000 genes, filtering out cells with mitochondrial content > 20%, scaling based on UMI count, mitochondrial read content, principal component analysis, unsupervised clustering based on significant pca components, tSNEplot visualization and differential expression analysis using Wilcox test. The library mRNA prep is sequenced on an Illumina HiSeq 4000 platform. The output from the sequencer is then analyzed using the 10XGenomics software CellRanger. This step includes the alignment of the reads to human genome (GRCH38). The raw file output from CellRanger is further processed using Seurat R package. The downstream processing includes: Log transformation, filtering out cells with < 500 genes (or < 400 UMI) and > 5000 genes, filtering out cells with mitochondrial content > 20%, scaling based on UMI count, mitochondrial read content, principal component analysis, unsupervised clustering based on significant pca components, tSNEplot visualization and differential expression analysis using Wilcox test.