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Working

Microwell-based Single-Cell RNA-seq

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Human Cell Atlas Method Development Community

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ABSTRACT

(**On**-chip reverse transcription version, Sims Lab – Jinzhou Yuan and Yim L. Cheng) 7/6/2017

Microfluidic Single-Cell RNA-seq-On-chip-reverse-transcription-Sims_Lab-YLC_clean.docx

GUIDELINES

Our single-cell RNA-seq technology uses a custom microfluidic device that we produce in our lab.

The CAD drawings of these devices that one can submit to a foundry for photolithographic production of molds from which these devices can be routinely generated:

http://www.columbia.edu/~pas2182/localhost/protocols/First_Mold_JY_CU_6inchwafer_microwellarrays_50umthick.dwg

http://www.columbia.edu/~pas2182/localhost/protocols/Second_Mold_JY_CU_6inchwafer_flowcell_100umthick.dwg

SAFETY WARNINGS

Please refer to the SDS (Safety Data Sheet) for information about hazards, and to obtain advice on safety precautions.

BEFORE STARTING

Day before experiment:

Device Preparation

- Fill a new device with wash buffer (20mM Tris-HCl pH7.9, 50mM NaCl, 0.1% Tween 20). Cover both the inlet and outlet of the device with a puddle of wash buffer.
- Store the wash buffer filled device in a humid chamber (P1000 pipette tip container filled with water) at room temperature.

Experiment day 1

1 **Live stain cells**

Harvest and resuspend 500,000 cells in **1 ml TBS (500 cells/uL).**

2 Incubate the cells in 4 uM CalceinAM (4ul 1mM CalceinAM per ml cells) for **00:15:00** on ice.

3 **Preparation of lysis buffer, and wash buffer, and fluorinated oil**

While the cells are being live stained, make

1 ml lysis buffer (**990 µl buffer TCL** + **10 µl 2-Mercaptoethanol**)

2 ml RNase inhibitor-doped wash buffer (**1998 µl wash buffer** + **2 µl SUPERaseIN**).

4 Load buffers and fluorinated oil to reagent reservoirs

- reagent reservoir 1: **1 ml lysis buffer**
- reagent reservoir 4: **1.8 ml fluorinated oil**
- reagent reservoir 5: **2 ml RNase inhibitor-doped wash buffer**

5 **Prepare mRNA-capture-beads**

Gently vortex the beads, transfer **500 µl** of beads into a microcentrifuge tube.

6 Wash the beads with **1 ml TBS buffer** and resuspend the beads in **400 µl TBS buffer** . Keep the beads on ice before use.

7 **mRNA-capture-bead and cell loadings**

Flush **1 ml TBS** through the wash buffer-filled device. Exercise caution to avoid flowing air into the device.

8 Load **500 µl live-stained cells** into the TBS-flushed device. Fine tune the size of liquid puddles on both inlet and outlet of the device until the cells stop moving in the device. Let cells settle for **00:03:00** .

9 Flush **1 ml TBS** through the cell-loaded device. Flush gently so that the loaded cells don't get washed out.

10 Confirm 5-10% of wells contain single cell under microscope.

11 Load **400 µl TBS-washed beads** into the device. Reuse the beads to repeat the loading until >60% wells contain beads. Load gently so that the bead flow does not wash cells out.

12 Flush **1 ml TBS** through the cell-bead-loaded device. Flush gently so that the loaded cells and beads don't get washed out.

13 Confirm >60% of wells contain mRNA-capture-bead under microscope.

14 **Pre-run fluidics wash**

Open channel 1 for **00:00:10** by executing the following command in the MinGW terminal: "**Valvesonly_3_dual_3.exe 10000 1**".

15 Open channel 4 for **00:00:10** by executing the following command in the MinGW terminal: "**Valvesonly_3_dual_3.exe 10000 4**".

16 Open channel 5 for **00:00:30** by executing the following command in the MinGW terminal: "**Valvesonly_3_dual_3.exe 30000 5**".

17 **Connect the device to the fluidics system and start the run**

Plug the inflow (red) and outflow (green) tubing to the inlet and outlet of the device respectively. **Make sure that the tubings are NOT inserted all the way to the bottom of the inlet or outlet as this can clog the device**, which will prevent liquid from flowing through or flow at a significantly reduced flow rate.

18 Start the run by executing the following commend in the MinGW terminal: "**SingleCellRNACapture_TCL_dual_large_array_3.exe**". Lysis buffer will be pushed through the device immediately followed by fluorinated oil, which will seal the wells, physically isolating the lysate of each individual cell.

19 The device will be heated to **50 °C** to promote cell lysis and mRNA capture, and then cooled back to room temperature.

20 **Cell lysis QC and mRNA capture**

Once the device has been cooled to room temperature, disconnect the device from the fluidics system. Seal both inlet and outlet of the device with disc tapes.

21 Imaging the device under fluorescence microscope with a 10X objective. Check the distribution of lysate (traced by the live stain dye) in the device. You should see the fluorescent dye fill up the wells that had cells, and the absence of fluorescent dye in wells that did not have cell. If this is the case, unseal the inlet and outlet of the device, reconnect the device to the fluidics system, and proceed to the next step. Otherwise, abort the experiment.

22 Reverse transcription

Prepare the following reverse transcription mixture

Final Concentration	Volume	Stock Concentration
1X Maxima RT buffer	100 µL	5X Maxima RT buffer
1 mM dNTPs	50 µL	10 mM dNTP
1 U/ul SuperaseIN	25 µL	20 U/ul SuperaseIN
2.5 uM SMRT_TSO	12.5 µL	100 uM SMRT_TSO
10 U/ul Maxima H- RT	25 µL	200 U/ul Maxima H- RT
0.1% Tween 20	5 µL	10% Tween20
Nuclease free water	282.5 µL	
Total	500 µL	

23 Load all **500 µl reverse transcription mixture** to reagent reservoir 2 before mRNA capture step is completed.

24 **Bead extraction**

Assemble a syringe-tubing set: 2.5 cm green PEEK tubing and 1ml syringe connected with 12 cm tygon tubing.

25 Prepare **3 ml wash buffer** , keep on ice

26 "Massage" the device to dislodge the beads from the device, then flow **1 ml wash buffer** through the device with syringe-tubing set, **COLLECT the overflow**.

cDNA-coated beads will be washed out from the device with the buffer flow, so you want to collect the overflow.

27 Repeat step 26 until >95% of the beads have been removed from the device.

go to step #26 **Repeat step 26**

28 Wash the cDNA-beads with

1 ml TE/SDS buffer (10 mM Tris pH 8.0, 1 mM EDTA, 0.5% SDS)

29 Wash the cDNA-beads with

1 ml TE/TW buffer (10 mM Tris pH 8.0, 1 mM EDTA, 0.01% Tween-20) (1/2)

30 Wash the cDNA-beads with

1 ml TE/TW buffer (10 mM Tris pH 8.0, 1 mM EDTA, 0.01% Tween-20) (2/2)

31 Resuspend the cDNA-beads in **1 ml TE/TW buffer** .

32 Store cDNA-beads at **4 °C** .

33 **Wash fluidics system**

Rinse and fill ALL reagent reservoirs (except reservoir 4) with DI water.

34 Execute the following command in the MinGW terminal: "**SingleCellRNACapture_wash_dual_3.exe**"

Experiment day 2

35 **Exo-I reaction**

Prepare the following Exo-I reaction mixture

Final Concentration	Volume	Stock Concentration
1X Exo I buffer	5 µL	10X Exo I buffer
1 U/uL Exo I	2.5 µL	20 U/uL
Nuclease free water	42.5 µL	
Total	50 µL	

36 Wash the cDNA-beads with **1 ml nuclease-free water** . (1/2)

37 Wash the cDNA-beads with **1 ml nuclease-free water** . (2/2)

38 Resuspend the beads in **50 µl Exo-I reaction mixture** .

39 Run the following program on a thermocycler:

Temperature	Time	Number of cycles
37 °C	30 minutes	1
4 °C	Hold	

40 Wash the ExoI-treated cDNA-beads with **1 ml TE/SDS buffer** .

41 Wash the ExoI-treated cDNA-beads with **1 ml TE/TW buffer** . (1/2)

42 Wash the ExoI-treated cDNA-beads with **1 ml TE/TW buffer** . (2/2)

43 Wash the ExoI-treated cDNA-beads with **1 ml nuclease-free water** . (1/2)

44 Wash the ExoI-treated cDNA-beads with **1 ml nuclease-free water** . (2/2)

45 **SMRT PCR reaction (cDNA PCR amplification)**

Prepare the following SMRT PCR reaction mixture

Final Concentration	Volume	Stock Concentration
1X PCR Ready Mix	500 µL	2X PCR Ready Mix
1 uM SMRT PCR primer	10 µL	100 uM SMRT PCR primer
Nuclease free water	490 µL	
Total	1000 µL	

46 Resuspend cDNA-beads in **1000 µl SMRT PCR reaction mixture** . Aliquot **50 µl** per PCR tube.

47 Run the following PCR program on a thermocycler:

Temperature	Time	Number of cycles
95 °C	3 minutes	1
98 °C	20 seconds	4
65 °C	45 seconds	
72 °C	3 minutes	
98 °C	20 seconds	8
67 °C	20 seconds	
72 °C	3 minutes	
72 °C	5 minutes	1
4 °C	hold	

48 **SMRT PCR product purification with 0.6X Ampure beads**

Combine all SMRT PCR products into a single microcentrifuge tube.

49 Spin for **00:01:00** , transfer **900 µl supernatant** to a new microcentrifuge tube.

50 Add **540 µl Ampure beads** (0.6X volume) to tube. Incubate at room temperature for **00:08:00** .

51 Place tube on a magnetic stand for **00:05:00** . Discard the supernatant.

52 Wash Ampure beads with 80% ethanol twice

- Add **1 ml freshly-made 80% ethanol in nuclease-free water** to tube, wait for **00:00:30** , remove 80% ethanol.
- Repeat 80% ethanol wash.
- Quick spin for **00:00:30** , place it back on a magnetic stand for **00:00:30** , and remove residual 80% ethanol.

53 Cover the tube with a KimWipe, air dry the Ampure beads on the magnetic stand for **00:05:00** .

54 Elute cDNA off Ampure beads with 20 ul nuclease-free water

- Add **20 µl nuclease-free water** directly on the Ampure beads, remove the tube from the magnetic stand, mix by pipetting.
- Incubate at room temperature for **00:05:00** .
- Place tube on a magnetic stand for **00:03:00** .
- Keep supernatant (amplified cDNA).

55 Store cDNA at **-20 °C** .

56 cDNA QC with Qubit and Bioanalyzer (follow vendor's exact instructions).

57 **Nextera tagmentation reaction (Nextera XT kit)**

Add **10 µl TD Buffer** to a PCR tube.

58 Add **5 µl** of 0.6 ng cDNA plus nuclease free water.

59 Add **5 µl ATM** to the PCR tube. Mix by pipetting.

60 Run the following program on a thermocycler:

Temperature	Time	Number of cycles
55 °C	5 minutes	1
10 °C	hold	

61 Immediately after the temperature reaches 10 °C, add **5 µl NT Buffer** to the PCR tube and mix by pipetting.

62 Incubate at room temperature for **00:05:00** .

63 **Selective amplification of the 3' end fragment of cDNA (Nextera XT kit)**

Add the following reagents to the end product in step 62 and mix by pipetting:

Reagent	Volume
NPM	15 µL
N7 PCR primer	5 µL
2 uM Custom P5 primer (not from Nextera XT kit)	5 µL

64 Run the following program on a thermocycler:

Temperature	Time	Number of cycles
72 °C	3 minutes	1
95 °C	30 seconds	1
95 °C	10 seconds	12
55 °C	30 seconds	
72 °C	30 seconds	
72 °C	5 minutes	1
4 °C	hold	

65 **Nextera PCR product purification with 0.6X + 1X Ampure bead**

Quick spin the Nextera PCR product **00:01:00** , transfer **50 µl PCR product** to a new microcentrifuge tube.

66 Add **30 µl Ampure beads** (0.6X volume) to tube. Incubate at room temperature for **00:08:00** .

67 Place tube on a magnetic stand for **00:05:00** . Discard the supernatant.

68 Wash Ampure beads with 80% ethanol twice

- Add **200 µl freshly-made 80% ethanol in nuclease-free water** to tube, wait for **00:00:30** , remove 80% ethanol.
- Repeat 80% ethanol wash.
- Quick spin for **00:00:30** , place it back on a magnetic stand for **00:00:30** , and remove residual 80% ethanol.

69 Cover the tube with a KimWipe, air dry the Ampure beads on the magnetic stand for **00:05:00** .

70 Elute the Nextera library off Ampure beads with 50 ul nuclease-free water

- Add **50 µl nuclease-free water** directly on the Ampure beads, remove the tube from the magnetic stand, mix the beads and the water by pipetting.
- Incubate at room temperature for **00:05:00** .
- Place tube on a magnetic stand for **00:03:00** .
- Keep supernatant (amplified cDNA).

71 Repeat Ampure bead purification steps (steps 65-70) with 1x volume Ampure bead (50 ul) and elute the Nextera library off Ampure beads with **20 µl nuclease-free water** .

go to step #65

72 Store the Nextera library at **-20 °C** .

73 Nextera library QC with Qubit and Bioanalyzer (follow vendor's exact instructions).

74 **Sequencing**

Use custom read 1 sequencing primer.
Use 20% phix.
21 cycles on read 1.
63 cycles on read 2.
8 cycles on index read 1 (if multiple samples are pooled together).