**Microwell-based Single-Cell RNA-seq**

 (**Off**-chip reverse transcription version, Sims Lab – Jinzhou Yuan and Yim L. Cheng) 6/20/2017

**Day before experiment:**

1. Device Preparation
	1. 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.
	2. 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
	1. Harvest and resuspend 500,000 cells in 1 mL TBS **(500 cells/uL)**
	2. Incubate the cells in 4uM CalceinAM (4ul 1mM CalceinAM per ml cells) for 15 minutes on ice.
2. Preparation of lysis buffer, and wash buffer, and fluorinated oil
3. While the cells are being live stained, make

 1 mL lysis buffer (990 uL buffer TCL + 10 uL 2-Mercaptoethanol)

 2 mL RNase inhibitor-doped wash buffer (1998 uL wash buffer + 2 uL SUPERaseIN).

1. Load buffers and fluorinated oil to reagent reservoirs
	* reagent reservoir 1: 1ml lysis buffer
	* reagent reservoir 4: 1.8ml fluorinated oil
	* reagent reservoir 5: 2ml RNase inhibitor-doped wash buffer
2. Prepare mRNA-capture-beads
3. Gently vortex the beads, transfer 500 uL of beads into a microcentrifuge tube.
4. Wash the beads with 1 mL TBS buffer and resuspend the beads in 400 uL TBS buffer. Keep the beads on ice before use.
5. mRNA-capture-bead and cell loadings
6. Flush 1 mL TBS through the wash buffer-filled device. Exercise caution to avoid flowing air into the device.
7. Load 500 uL 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 3 minutes.
8. Flush 1 mL TBS through the cell-loaded device. Flush gently so that the loaded cells don’t get washed out.
9. **Confirm 5-10% of wells contain single cell under microscope**
10. Load 400 uL 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.
11. Flush 1 mL TBS through the cell-bead-loaded device. Flush gently so that the loaded cells and beads don’t get washed out.
12. **Confirm >60% of wells contain mRNA-capture-bead under microscope**
13. Pre-run fluidics wash
14. Open channel 1 for 10 seconds by executing the following command in the MinGW terminal: “**Valvesonly\_3\_dual\_3.exe 10000 1**”.
15. Open channel 4 for 10 seconds by executing the following command in the MinGW terminal: “**Valvesonly\_3\_dual\_3.exe 10000 4**”.
16. Open channel 5 for 30 seconds 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
18. 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.
19. Start the run by executing the following commend in the MinGW terminal: “**SingleCellRNACapture\_TCL\_dual\_large\_array\_3\_lysis\_only.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.
20. The device will be heated to 50 ºC to promote cell lysis and mRNA capture, and then cooled back to room temperature.
21. Cell lysis QC and mRNA capture
	1. 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.
	2. 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.

* 1. Transfer the sealed device to a humid chamber, which helps minimize liquid loss due to evaporation.
	2. Incubate for 90 minutes during which mRNA molecules are captured onto the beads by hybridization.
1. Wash fluidics system
	1. Rinse and fill ALL reagent reservoirs (except reservoir 4) with DI water
	2. Execute the following command in the MinGW terminal: “**SingleCellRNACapture\_wash\_dual\_3.exe**”
2. Bead extraction
3. syringe-tubing set: 2.5cm green PEEK tubing and 1ml syringe connected with 12cm tygon tubing.
4. Prepare 3ml RNase inhibitor-doped wash buffer, kept on ice
5. Gently flow 1 mL RNase inhibitor-doped wash buffer through the device using a pre-assembled syringe-tubing set, discard the overflow. This step will unseal the wells, remove perforated oil and uncaptured mRNA from the device.
6. “Massage” the device to dislodge the beads from the device, then flow 1 mL RNase inhibitor-doped wash buffer through the device with syringe-tubing set, **COLLECT the overflow**. mRNA-coated beads will be washed out from the device with the buffer flow, so you want to collect the overflow.
7. Repeat step d until >95% of the beads have been removed from the device.
8. Reverse transcription
	1. Prepare the following reverse transcription mixture

|  |  |  |
| --- | --- | --- |
| **Final Concentration** | **Volume** | **Stock Concentration** |
| 1X Maxima RT buffer | 40 uL | 5X Maxima RT buffer |
| 1 mM dNTPs | 20 uL | 10mM dNTP |
| 1 U/ul SuperaseIN | 10 uL | 20U/ul SuperaseIN |
| 2.5 uM SMRT\_TSO | 5 uL | 100uM SMRT\_TSO |
| 10 U/ul Maxima H- RT | 10 uL | 200U/ul Maxima H- RT |
| 0.1% Tween 20 | 2 uL | 10% Tween20 |
| Nuclease free water | 113 uL |  |
| Total | 200 uL |  |

* 1. Wash the mRNA-captured-beads with 1 mL of RNase inhibitor-doped wash buffer and 1 mL of nuclease-free water twice.
	2. Re-suspend the beads in 200 uL reverse transcription mixture, aliquot 50ul per PCR tubes
	3. Run the following program on a thermocycler:

|  |  |  |
| --- | --- | --- |
| **Temperature** | **Time** | **Number of cycles** |
| 25 °C | 30 minutes | 1 |
| 42 °C | 90 minutes | 1 |
| 4 °C | hold |  |

* 1. Combine cDNA-beads in one microcentrifuge tube
	2. Wash the cDNA-beads with
* 1 mL of TE/SDS buffer (10 mM Tris pH 8.0, 1 mM EDTA, 0.5% SDS)
* 1 mL of TE/TW buffer twice (10 mM Tris pH 8.0, 1 mM EDTA, 0.01% Tween-20)
	1. Resuspend the cDNA-beads in 1 mL of TE/TW buffer.
	2. Store cDNA-beads at 4 ºC

**Experiment day 2:**

1. Exo-I reaction
2. Prepare the following Exo-I reaction mixture

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

1. Wash with 1 mL of nuclease-free water twice
2. Resuspend the beads in 50 uL of the Exo-I reaction mixture.
3. Run the following program on a thermocycler:

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

1. Wash the ExoI-treated cDNA-beads with
* 1 mL of TE/SDS buffer
* 1 mL of TE/TW buffer twice
* 1 mL of nuclease-free water twice
1. Re-suspend the cDNA-beads in 1 mL of nuclease-free water.
2. SMRT PCR reaction (cDNA PCR amplification)

a. Prepare the following SMRT PCR reaction mixture

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

1. Resuspend cDNA-beads in 1000 uL SMRT PCR reaction mixture. Aliquot 50ul per PCR tube
2. Run the following PCR program on a thermocycler:

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

1. SMRT PCR product purification with 0.6X Ampure beads
	1. Combine all SMRT PCR products into a single microcentrifuge tube
	2. Spin for 1 minute, transfer 900 uL supernatant to a new microcentrifuge tube.
	3. Add 540 uL (0.6X volume) of Ampure beads to tube. Incubate at room temperature for 8 minutes.
	4. Place tube on a magnetic stand for 5 minutes. Discard the supernatant.
	5. Wash Ampure beads with 80% ethanol twice
		* Add 1 mL freshly-made 80% ethanol in nuclease-free water to tube, wait for 30 seconds, remove 80% ethanol
		* Repeat 80% ethanol wash
		* quick spin for 30 seconds, place it back on a magnetic stand for 30seconds, and remove residual 80% ethanol
	6. Cover the tube with a KimWipe, air dry the Ampure beads on the magnetic stand for 5 minutes.
	7. Elute cDNA off Ampure beads with 20 ul nuclease-free water
* Add 20 uL nuclease-free water directly on the Ampure beads, remove the tube from the magnetic stand, mix by pipetting.
* Incubate at room temperature for 5 minutes.
* Place tube on a magnetic stand for 3 minutes
* Keep supernatant (amplified cDNA)
	1. Store cDNA at -20 ºC

14.5 cDNA QC with Qubit and Bioanalyzer (follow vendor’s exact instructions)

1. Nextera tagmentation reaction (Nextera XT kit)
	1. Add 10 uL of TD Buffer to a PCR tube.
	2. Add 5 uL of 0.6ng cDNA plus nuclease free water
	3. Add 5 uL of ATM to the PCR tube. Mix by pipetting.
	4. Run the following program on a thermocycler:

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

* 1. Immediately after the temperature reaches 10 ºC, add 5 uL of NT Buffer to the PCR tube and mix by pipetting.
	2. Incubate at room temperature for 5 minutes.
1. Selective amplification of the 3' end fragment of cDNA (Nextera XT kit)
2. Add the following reagents to the end product in step 15 and mix by pipetting:

|  |  |
| --- | --- |
| **Reagent** | **Volume** |
| NPM  | 15 uL |
| N7 PCR primer  | 5 uL |
| 2 uM Custom P5 primer (not from Nextera XT kit) | 5 uL |

1. 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 |  |
| 55 °C | 30 seconds | 12 |
| 72 °C | 30 seconds |  |
| 72 °C | 5 minutes | 1 |
| 4 °C | hold |  |

1. Nextera PCR product purification with 0.6X + 1x Ampure bead
	1. Quick spin the Nextera PCR product 1 minute, transfer 50ul PCR product to a new microcetrifuge tube.
	2. Add 30 uL (0.6X volume) of Ampure beads to tube. Incubate at room temperature for 8 minutes.
	3. Place tube on a magnetic stand for 5 minutes. Discard the supernatant.
	4. Wash Ampure beads with 80% ethanol twice
		* Add 200ul freshly-made 80% ethanol in nuclease-free water to tube, wait for 30 seconds, remove 80% ethanol
		* Repeat 80% ethanol wash
		* Quick spin for 30 seconds, place it back on a magnetic stand for 30seconds, and remove residual 80% ethanol
	5. Cover the tube with a KimWipe, air dry the Ampure beads on the magnetic stand for 5 minutes.
	6. Elute the Nextera library off Ampure beads with 50 ul nuclease-free water
* Add 50 uL 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 5 minutes.
* Place tube on a magnetic stand for 3 minutes
* Keep supernatant (amplified cDNA)
	1. Repeat Ampure bead purification steps with 1x volume Ampure bead (50ul) and elute the Nextera library off Ampure beads with 20 ul nuclease-free water
	2. Store the Nextera library at -20 ºC

17.5 Nextera library QC with Qubit and Bioanalyzer (follow vendor’s exact instructions)

18. Sequencing

 Use custom read 1 sequencing primer.

 Use 40% phix.

 26 cycles on read 1.

 58 cycles on read 2.

 8 cycles on index read 1 (if multiple samples are pooled together).