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High-throughput single cell sequencing using CEL-Seq2 on a nanoliter dispensing robot

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Abstract

For the detection of subtle transcriptome changes reflecting fate biases, an scRNA-seq method is required that maximizes both sensitivity and accuracy. Recent benchmarking has demonstrated that CEL-Seq2 optimized both of these factors, but comes with high costs per cell (Hashimshony, T. et al. Genome Biol. 17, 77, 2016; Svensson, V. et al. Nat. Methods 14, 381–387, 2017). To enable high-throughput sequencing by the CEL-Seq2 method we here establish a robotic version of this method that permits sequencing of thousands of cells at substantially reduced costs.

Introduction

Studying tissues and organs at the single cell level offers the possibility of unprecedented insights into the differentiation dynamics and the cellular heterogeneity of the tissue or organ of interest. In this protocol we describe a down-scaled version of CEL-Seq2 for the use with a nanoliter pipetting robot that allows us to sequence thousands of cells at low cost.

Reagents

1. SuperScript II™ Reverse Transcriptase (Invitrogen, 18064014)
2. RNaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen, 10777019)
3. Second Strand Buffer (Invitrogen, 10812014)
4. E. coli DNA Polymerase I (Invitrogen, 18010025)
5. E. coli DNA Ligase (Invitrogen, 18052019)
6. E. coli RNase H (Invitrogen, 18021071)
7. MEGAscript T7 Transcription Kit (Invitrogen, AM1334)
8. ExoSAP-IT For PCR Product CleanUp (Applied Biosystems™, 75001.1.ML)
9. NEBNext® Magnesium RNA Fragmentation Module (New England Biolabs, E6150S)
10. Agencourt® AMPure XP beads (Beckman Coulter, A63881)
11. Agencourt® RNAclean XP beads (Beckman Coulter, A63987)
12. Agilent RNA 6000 Pico Kit
13. Phusion® High-Fidelity PCR Master Mix with HF Buffer (New England Biolabs)
14. 10 µM randomhexRT primer (GCCTTGGCACCCGAGAATTCCANNNNNN)

15. 10 µM Illumina RNA PCR (RP) primers
16. Hydrophobic encapsulation barrier (Vapor-Lock, Qiagen, 981611 or Mineral oil, Sigma, M8410)
17. Triton® X-100 (Sigma, T8787)
18. Qubit dsDNA HS (High Sensitivity) Assay Kit
19. Agilent High Sensitivity DNA kit
20. 192 barcoded primers

Equipment

1. mosquito®HTS (TTP Labtech)
2. Cell sorter capable of sorting into 384-well plates (e.g. BD Influx™, BD FACSAria™ Fusion)
3. Laboratory centrifuge suitable for well plates
4. Mini-centrifuge to spin tubes
5. 384 and 96-well plates, LoBind, DNase/RNase-free
6. Aluminium plate sealers, temperature range: -80ºC to 105ºC
7. 384-well and 96-well thermal cycler or hybridization oven and fridge with adjustable temperature (16˚C)
8. 96-well plate magnetic stand for bead clean-up
9. Qubit® Fluorometer (Invitrogen)
10. Agilent 2100 Bioanalyzer
11. DNA LoBind tubes, PCR Clean – 0.5 ml, 1.5 ml and 2 ml
12. DNA Low Binding filter tips, DNase and RNase-free
13. RNase decontamination solution
14. RNase-free water
15. RNase-free ethanol, molecular biology grade

16. Single-channel and multi-channel pipettes

Procedure

General Remarks:

Before starting the experiment make sure to clean your bench and pipets using RNase decontamination solution. Use RNase-free DNA-low binding tubes and tips. Each time well plates are centrifuged they should be covered using aluminium plate sealers. Perform all centrifugation steps at 4˚C.

Plate preparation for single cell sorting

The following steps describe how to prepare 8 x 384-well plates for single cell sorting.

Prepare barcoded primer source plate:

1. Prepare a mastermix of the following reagents (enough for 8 plates): a. 840 µl water with 0.35% Triton® X-100

b. 120 µl 10mM dNTP

c. 240 µl water with 1:100000 ERCC mix 1 or 2

2. Pipet 5 µl of the solution into each of the 192 wells of a 384-well plate using a multi-channel pipet (columns 1-12, rows A-P). Avoid bubbles.
3. Centrifuge the plate at 2200g for 1 min
4. Dispense 1000 nl of each 192 barcoded primers at 1µM working concentration from a 384-well plate into the prepared 384-well plate containing 5 µl buffer using the mosquito®HTS. (mosquito from here on)
5. Add a mixing routine for every dispense step in the program (see Advanced Options for dispense step) and mix the solution 10 times and by moving the tips up by 2 mm.
6. Centrifuge the plate at 2200g for 1 min

Preparation of a source plate containing hydrophobic encapsulation barrier (Vapor-Lock)

7. Pipet 25µl of Vapor-Lock into each of the 192 wells of a 384-well plate
8. Centrifuge the plate at 2200g for 1 min

Preparation of plates for single cell sorting

9. Place the primer source plate into the first plate position of the mosquito
10. Put 4 empty plates into the remaining bays.
11. Dispense 240 nl of primer mix from the first column of your primer source plate into each first column and column 13 of your empty plates
12. Change the mosquito pipets
13. Dispense the remaining columns in the same manner and change the mosquito pipets every time you dispense into a new column i.e. dispense from column 2 of the source plate to columns 2 and 14 in the empty plates, from column 3 of the source plate to columns 3 and 15 in the empty plates etc. Like this one of the 192 barcodes is used twice per 384-well plate.
14. After the primers are dispensed change the primer source plate with the plate containing Vapor-Lock and dispense 1.2 µl of Vapor-Lock in the same manner, i.e. dispense 1.2 µl from columns 1, 2, 3. to columns 1 and 13, 2 and 14, 3 and 15 etc. of the plates containing the primer mix. Change pipets when pipetting from a new column of the source plate.
15. Centrifuge the plates at 2200g for 1 min and freeze them at -20˚C until they are used. (Note: Instead of using Vapor-Lock one can use mineral oil. In this regard it is possible to first dispense 1.2 µl mineral oil into every well of the empty 384-well plates without changing the pipets and then dispense the primer mix as described in steps 11. - 13.)

Cell Sorting

16. Use an appropriate nozzle size for your cells of interest (min. 5 times the cell size, 100µm nozzles can be used if the sorted cells are heterogenous in size)
17. Include dead live staining, if possible, especially with extended cell extraction procedures.

18. Exclude doublets using FSC-W to FSC-H and SSC-W to SSC-H ratios as well as the single cell mode of the cell sorter.

19. Centrifuge the plates at 2200g for 10 min at 4˚C and store them at -80˚C until further processing using CEL-Seq2.

Amplified RNA preparation from single cells

The following steps describe how to prepare one 384-well plate using mCEL-Seq2.

20. Incubate the plate containing your cells (sample plate) at 90°C for 3 min in a thermocycler (lid: 105°C)

21. Centrifuge the plates at 2200g for 1 min at 4˚C and transfer plates on ice.

Reverse transcription

22. Prepare a mastermix for the reverse transcriptase reaction (the receipe includes additional volume to account for dead volume in the mosquito pipet tips): a. First Strand Buffer 70 µl

b. DTT 0.1 M 35 µl

c. RNaseOut 17.5 µl

d. Superscript II 17.5 µl

total 140 µl

23. Pipet 8.5 µl into each well of one column of a new 384-well plate and centrifuge the plate briefly. This plate will serve as your buffer source plate.

24. Dispense 160 nl of First Strand Reaction mix from the buffer source plate to every column of your sample plate and change the pipets after every dispense step.

25. Centrifuge the plates at 2200g for 1 min at 4˚C

26. Incubate the sample plate at 42˚C for 1h (lid: 50˚C).

27. Heat-inactivate the reverse transcriptase at 70˚C for 10 min.

28. Chill the plate on ice and centrifuge the plates at 2200g for 1 min at 4˚C. Second Strand Synthesis

29. Prepare a mastermix for second strand synthesis: a. Ultrapure water: 631.4 µl

b. Second Strand Buffer: 205 µl

c. dNTP 20.5 µl

d. E. coli Ligase 7.38 µl

e. E. coli DNA Polymerase 28.7 µl

f. RNase H 7.38 µl

total	900.36 µl

30. Pipet 14 µl into each well of four new columns of your buffer source plate. This approach is used when pooling 96 cells per library (see below). (Alternatively it is possible to pipet 28 µl into each well of 2 columns when 192 cells are to be pooled into one library.)

31. Dispense 2196 nl Second Strand Reaction mix in two steps (2 x 1098 nl) from the buffer source plate into every well of your sample plate.

32. Pipets have to be changed four times after dispensing into columns 1-6, 7-12, 13-18, 19-24. This is the case when 96 cells should be pooled together. (If 192 cells should be pooled, change pipets after dispensing into columns 1-12 and13-24)

33. Centrifuge the sample plate at 2200g for 1 min at 4˚C

34. Incubate the sample plate at 16˚C for 2 h.

cDNA cleanup

35. Prewarm AMPure XP beads to room temperature (takes app. 30 min)

36. Vortex beads until dispersed

37. Pool columns 1-6, 7-12, 13-18, 19-24 each into a single tube. Pool the columns that

should be pooled into a single column using a multichannel pipet and then transfer the contents of this column into a tube.

38. Centrifuge the four tubes at 10000g for 1 min to separate the aqueous phase from the oil.
39. For every sample transfer the aqueous phase (app. 200 µl) into two wells (app. 100 µl each) of a DNA low-binding 96-well plate.
40. Add 0.8 volumes of AMPure XP beads to every well containing a sample.
41. Mix by resuspending 5 times using a multichannel pipet
42. Incubate at room temperature for 10 min
43. Place on magnetic stand and let the solution clear
44. Remove and discard the supernatant
45. Add 180 µl of freshly prepared 80% ethanol
46. Incubate 40 seconds and remove the ethanol completely
47. Repeat the washing step once
48. Air dry beads for 10 min
49. Resuspend the sample in the two wells with a total of 7 µl water.
50. Incubate at room temperature for 2 min
51. Place on magnetic stand until solution is clear
52. Transfer app. 6.4 µl of the supernatant into a fresh PCR tube In vitro transcription
53. Add 9.6 µl of in vitro transcription mix, consisting of 1.6 µl of ATP, GTP, CTP, UTP, 10x T7 Buffer and T7 enzyme each (mastermix: 7 µl of every component). Mix well.
54. Incubate at 37˚C for 13 h (lid:70˚C)

aRNA cleanup

55. Add 6 µl EXO-SAP to every sample and incubate at 37˚C for 15 min
56. Add 2.44 µl 10x Fragmentation Buffer and mix

57. Incubate at 94˚C for 3min

58. Immediately after move the samples to ice and add 2.44 µl 10x Fragmentation Stop Buffer

59. Prewarm RNAClean XP beads to room temperature (takes app. 30 min)

60. Vortex beads until dispersed

61. Add 21.5 µl of beads to every sample and mix well

62. Incubate at room temperature for 10 min

63. Place on magnetic stand and let the solution clear

64. Remove and discard the supernatant

65. Add 180 µl of freshly prepared 70% ethanol

66. Incubate 40 seconds and remove the ethanol completely

67. Repeat wash 2 times

68. Air dry beads for 10 min

69. Resuspend each amplified RNA (aRNA) sample with 7 µl water.

70. Perform a quality check using 1µl of aRNA using the Agilent RNA 6000 Pico Kit and the Bioanalyzer. The average fragment size of the aRNA should be between 500 bp (± 50 bp).

Library preparation

Reverse Transcription of aRNA

71. Add 1.5 µl of the following solution to 5 µl of aRNA: a. 1 µl random Hexamers (5 x MM: 5 µl)

b. 0.5 µl dNTP (5 x MM: 2.5 µl)

72. Incubate at 65˚C for 5 min and chill on ice thereafter

73. Add 4 µl of First Strand Synthesis Solution a. 2 µl First Strand Buffer (5 x MM: 10 µl)

b. 1 µl 0.1M DTT (5 x MM: 5 µl)

c. 0.5 µl RNaseOUT (5 x MM: 2.5 µl)

d. 0.5 µl Superscript II (5 x MM: 2.5 µl)

74. Incubate at 25˚C for 10 min

75. Incubate at 42˚C for 1 h (lid: 50˚C)

PCR amplification

76. Add to every sample 2 µl of one uniquely indexed Illumina RPI primer

77. Add to every sample 38 µl of the following solution and mix: a. Ultra pure water 11 µl

b. Phusion HF PCR Master Mix 25 µl

c. Illumina primer RP1 2 µl

78. Amplify DNA in a PCR cycler using the following conditions: Denaturation

98˚C 30 s

11 cycles (up to 15 cycles with low aRNA concentration)

98˚C 10 s

60˚C 30 s

72˚C 30 s

Final extension

72˚C 10 min

4˚C ∞

1st Bead Cleanup of PCR products

79. Transfer samples (each 50 µl into a low binding 96-well plate

80. Prewarm AMPure XP beads to room temperature (takes app. 30 min)

81. Vortex beads until dispersed

82. Add 50 µl to each 50 µl PCR reaction

83. Incubate at room temperature for 10 min

84. Place on magnetic stand and let the solution clear

85. Remove supernatant

86. Wash with 180 µl freshly prepared 80% EtOH

87. Incubate for 40 s and discard supernatant without disturbing the beads
88. Repeat washing step once
89. Air dry beads for 10 min
90. Resuspend with 25 µl water, mix thoroughly
91. Incubate at room temperature for 2 min
92. Place on magnetic stand and let the solution clear
93. Transfer supernatant (25 µl) to a new well

2nd Bead Cleanup of PCR products

94. Prewarm AMPure XP beads to room temperature (takes app. 30 min)
95. Vortex beads until dispersed
96. Add 25 µl to each 25 µl solution
97. Incubate at room temperature for 10 min
98. Remove supernatant
99. Wash with 180 µl freshly prepared 80% EtOH
100. Incubate for 40 s, and discard supernatant without disturbing the beads
101. Repeat washing step once
102. Air dry beads for 10 min
103. Resuspend with 10 µl water, mix thoroughly
104. Incubate at room temperature for 2 min
105. Place on magnetic stand and let the solution clear
106. Transfer supernatant (10 µl) to a new tube
107. Library is ready for sequencing and can be stored at -20˚C
108. Measure concentration and fragment size of each library and pool equimolar amounts of libraries according to the used unique RPI guidelines (Illumina). Sequence cells at ~200000 reads per cell.

Timing

Preparation of plates for single cell sorting: 3 hours

Cell Sorting: 1 hour - 8 hours +

Reverse Transcription - in vitro transcription of cDNA: 1 day

RNA cleanup - Library preparation and purification: 1 day

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

celseq_primers.seq.txt