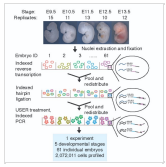

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sci-RNA-seq3

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2 Works for me dx.doi.org/10.17504/protocols.io.9yih7ue

Human Cell Atlas Method Development Community

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ABSTRACT

Single-cell combinatorial indexing ('sci-') is a methodological framework that employs split-pool barcoding to uniquely label the nucleic acid contents of large numbers of single cells or nuclei. Although the throughput of 'sci-' methods increases exponentially with the number of rounds of indexing, this potential has yet to be fully realized because of other factors such as the rate of cell loss and the limited reaction efficiency of some steps. To address this, we developed and extensively optimized 3-level sci-RNA-seq (sci-RNA-seq3), resulting in a workflow that can profile millions of cells per experiment. As previously multiple samples (e.g. replicates, timepoints, etc.) can be barcoded during the first round of indexing and concurrently processed.

EXTERNAL LINK

https://www.nature.com/articles/s41586-019-0969-x#citeas

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Cao, J., Spielmann, M. et al. The single-cell transcriptional landscape of mammalian organogenesis. Nature 566, 496–502 (2019) doi:10.1038/s41586-019-0969-x

ATTACHMENTS

sci3_primer_sequences.xls

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sci3_primer_sequences.xls

GUIDELINES

The protocol workflow is as follows:

1. Buffer preparation (Steps 1-5).
2. Nuclei extraction directly from tissues (~1.5 hours for 6 tissues) (Steps 6-10).
3. Nuclei fixation (~1.5 hours for 6 tissues) (Steps 11-16).
4. Nuclei permeabilization before reverse transcription (1 hour for 6 samples) (Steps 17-26).
5. Reverse transcription (~ 2 hours for 6 samples) (Steps 27-33).
6. Ligation (~ 2 hours) (Steps 34-44).
7. Second strand synthesis (~ 3 hours) (Steps 45-47).
8. Tagmentation (~10min) (Steps 48-50).
9. Ampure beads purification (~1 hour) (Steps 51-59).
10. PCR reaction and library preparation (~2 hours) (Steps 60-65).

It is important to start with a species-mixing experiment for validating the experimental setup is working. We normally start with a mixture of human (HEK293T) and mouse (NIH/3T3) cells. A good run normally yields single-cell transcriptomes with over 5000 UMIs (with over 20,000 sequencing reads) per cell and >98% purity.

Downstream analysis

The output of this protocol is the sequencing reads from Nova-seq or Next-seq, comprising i5 and i7 index reads (PCR barcode), read1 (RT barcode and ligation barcode) and read2 (cDNA sequence). The base calls are converted to fastq format using Illumina's bcl2fastq/v2.16 and demultiplexed based on PCR i5 and i7 barcodes using maximum likelihood demultiplexing package deML with default settings. Downstream sequence processing and single-cell digital expression matrix generation were done using a custom script on github.

Example dataset

We have deposited all of the raw data and processed data sets from the sci-RNA-seq3 experiment described in the Nature paper (Cao, J., Spielmann, M. et al. 2019) in Gene Expression Omnibus, accession GSE119945. The raw gene count matrix and processed data can be found in our website.

Required equipment:

Bioruptor Sonication device (diagenode, B01020001)
Bright-Line™ Hemacytometer (Sigma)
Centrifuge (cooled to 4°C) (Eppendorf, 5810R)
DynaMag™-96 Side Skirted Magnet (Thermo Fisher Scientific, 12027)
Eppendorf Mastercycler (thermal cycler)
FACs machine
Freezer (-20°C, -80°C) and refrigerator (4°C)
Gel box
Gel imager
Ice bucket
Liquid nitrogen tank for sample storage
Microscope
Multi-channel pipettes (10ul, 200ul) (Rainin Instrument)

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NextSeq 500 platform (Illumina)
Pipettors
Rainin Liquidator 96 Manual Pipetting System
Tissue culture hood
Tissue culture incubator

Primer sequences:
All primer sequences including RT/ligation/PCR primers are attached in the description section. All primers are ordered from IDT with standard desalting.

We will continue to add updates and increment the version number whenever we see opportunities to improve the protocol and revise the experimental steps.

MATERIALS TEXT

Nuclease free water (Ambion, AM 9937)
Corning 6 cm cell culture dish (Sigma Aldrich, Z707678-840EA)
Greiner Cellstar 6 well plates (Sigma Aldrich, M8562)
OEMTOOLS 25181 Razor Blades, 100 Pack (VWR, 55411-0055)
Falcon Cell Strainers 40 um (VWR, 10199-654)
SCIENCEWARE® Flowmi™ Cell Strainers for 1000µl Pipet Tips (VWR, Cat. 10204-924)
BD New STERILE , Sealed , 5 ML Syringes Only LUER Lock TIP, No Needle, Disposable (VWR, BD309646)
4% Paraformaldehyde (Formaldehyde) Aqueous Solution, EM Grade, 100 mL (Electron Microscopy Sciences 15-4-100)
SUPERase In RNase Inhibitor 20 U/uL (Thermo Fisher Scientific, AM2696)
BSA 20 mg/ml (NEB, B9000S)
1M Tris-HCl (pH 7.5) (Thermo Fisher Scientific, 15567027)
5M NaCl (Thermo Fisher Scientific, AM9759)
1M MgCl2 (Thermo Fisher Scientific, AM9530G)
IGEPAL CA-630 (Sigma-Aldrich, I8896-50ML)
Triton X-100 for molecular biology (Sigma Aldrich, 93443-100ML)
10mM dNTP (Thermo Fisher Scientific, R0192)
384 indexed oligo-dT primers (100uM, 5'- /5Phos/CAGAGCNNNNNNNN[10bp barcode]TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-3', where “N” is any base; IDT)
Superscript IV reverse transcriptase with 100mMDTT and buffer(Invitrogen, 18090200)
RNaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen, 10777019)
Quick ligation kit (NEB, M2200L)
384 indexed ligation primers (100uM, 5’- GCTCTG[9bp or 10bp barcode A]/ideoxyU/ACGACGCTCTTCCGATCT[reverse complement of barcode A]-3’)
Elution buffer (Qiagen, 19086)
NEBNext® Ultra II Non-Directional RNA Second Strand Synthesis Module (NEB, E7550S)
Nextera N7 adaptor loaded Tn5 (provided by Illumina)
DNA binding buffer (Zymo Research, D4004-1-L)
AMPure XP beads (Beckman Coulter, A63882)
USER enzyme (NEB, M5505L)
Ethanol (Sigma Aldrich, 459844-4L)
10 µM P5 primer (5'-AATGATACGGCGACCACCGAGATCTACAC[i5]ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3', IDT)
10 µM P7 primer (5'-CAAGCAGAAGACGGCATACGAGAT[i7]GTCTCGTGGGCTCGG-3', IDT)
NEBNext High-Fidelity 2X PCR Master Mix (NEB, M0541L)
Select-a-Size DNA Clean & Concentrator (Zymo Research, D4080)
Qubit dsDNA HS kit (Invitrogen, Q32854)
Qubit tubes (Invitrogen, Q32856)
6% TBE-PAGE gel (Invitrogen, EC6265BOX)
Quick-load 2-log DNA ladder (NEB, N0550L)
Nextseq V2 75 cycle kit (Illumina, FC-404-2005)
Falcon Tubes, 15 ml (VWR Scientific, 21008-936)
Falcon Tubes, 50 ml (VWR Scientific, 21008-940)
Green pack LTS 200ul filter tips (GP-L200F) (Rainin Instrument, 17002428)
Green pack LTS 20ul filter tips (GP-L20F) (Rainin Instrument, 17002429)
RT-L250WS wide-orifice LTS 250 ul (Rainin Instrument, 30389249)
Bright-Line™ Hemacytometer (Sigma Aldrich, Z359629-1EA)
DNA LoBind Tube 1.5 ml, PCR clean (Eppendorf North America, 22431021)

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LoBind clear, 96-well PCR Plate (Eppendorf North America, 30129512)
Reagent reservoirs (Fisher Scientific, 07-200-127)
Falcon® 5mL Round Bottom w/ Cell Strainer (Fisher Scientific, 352235)
Microseal 'B' Adhesive seal (Bio-Rad Laboratories, MSB1001)

ABSTRACT

Single-cell combinatorial indexing ('sci-') is a methodological framework that employs split-pool barcoding to uniquely label the nucleic acid contents of large numbers of single cells or nuclei. Although the throughput of 'sci-' methods increases exponentially with the number of rounds of indexing, this potential has yet to be fully realized because of other factors such as the rate of cell loss and the limited reaction efficiency of some steps. To address this, we developed and extensively optimized 3-level sci-RNA-seq (sci-RNA-seq3), resulting in a workflow that can profile millions of cells per experiment. As previously multiple samples (e.g. replicates, timepoints, etc.) can be barcoded during the first round of indexing and concurrently processed.

Buffer preparation

1 500ml Nuclei buffer (Stored in 4C): 10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 3 mM MgCl2 in nuclease-free water:

Reagent	Stock concentration	Final concentration	Volume (ml)
Tris-HCl (pH 7.5)	1M	10mM	5
NaCl	5M	10mM	1
MgCl2	1M	3mM	1.5
Nuclease-free water	NA	NA	492.5
Final volume			500

Filter the buffer through 0.22uM filter and store the buffer in 4C for up to 1 year.

2 20ml 10% (volume) IGEPAL CA-630 in nuclease-free water (stored in 4C).

Add 2ml IGEPAL CA-630 to 18ml nuclease-free water. Mix the solution by pipetting up and down for 20 times. The mix can be stored in 4C for up to 1 year.

3 20ml 10% (volume) Triton X-100 in nuclease-free water (stored in 4 degrees).

Add 2ml Triton X-100 to 18ml nuclease-free water. Mix the solution by pipetting up and down for 20 times. The mix can be stored in 4C for up to 1 year.

4 Cell lysis buffer(CLB) (Made fresh each time, 5ml per sample, stored on ice): Nuclei buffer with 0.1% IGEPAL CA-630, 1% SUPERase In RNase Inhibitor (20 U/µL, Ambion) and 1% BSA (20 mg/ml, NEB). For each sample, combine 4.85ml nuclei buffer, 50ul 10% (volume) IGEPAL CA-630, 50ul SUPERase In RNase Inhibitor (20 U/µL, Ambion), and 50ul BSA (20 mg/ml, NEB).

5 Nuclei suspension buffer (NSB) (Made fresh each time, 3ml per sample, stored on ice): Nuclei buffer with 1% SUPERase In RNase Inhibitor (20 U/µL, Ambion) and 1% BSA (20 mg/ml, NEB). For each sample, combine 4.9ml nuclei buffer, 50ul SUPERase In RNase Inhibitor (20 U/µL, Ambion), and 50ul BSA (20 mg/ml, NEB).

Nuclei extraction directly from tissues (~1.5 hours for 6 tissues)

6 Cool the centrifuge to 4C.
In a 6cm dish, add 1ml CLB to the frozen tissue (0.1g-0.5g, frozen in liquid nitrogen or -80C) for nuclei extraction. Cut the tissue into small pieces (< 1mm in diameter) with blade (for some hard tissues like heart, we can use surgical scalpel for better dissociation). If the tissue is in large chunk, we can mash the frozen tissue into powder with a hammer (on dry ice) before sampling the pulverized tissue for incubation with CLB.

7 Pipet the tissue up and down with 1ml pipet tip for 10 times (cut the top of the 1ml pipet tip for easy pipetting) and

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transfer the tissue into one 15ml tube with another 3ml CLB.

8 Transfer the CLB with tissue to the top of a 40um cell strainer (placed on the top of 1 well in 6-well plate on ice) and homogenize the tissue with the rubber tip of a syringe plunger. (During the tissue dissociation, make sure the tissue on the strainer is merged in cold lysis buffer).

9 Transfer the isolated and filtered nuclei into a new 15ml tube, pellet the nuclei (500g, 5min), and dump the supernatant.

10 Re-suspend the nuclei in 1ml CLB by pipetting up and down 3 times.

Nuclei fixation (~1.5 hours for 6 tissues)

11 Pellet the nuclei (500g 5min) and dump the supernatant.

12 Re-suspend the nuclei in 100ul NSB, add 10ml ice-cold 4% PFA, and invert the tubes for 5 times. Fix the nuclei on ice for 15min.

13 Pellet the nuclei (500g, 3min) and dump supernatant.

14 Re-suspend the nuclei with 1ml NSB and transfer the nuclei into a new tube. Pellet the nuclei (500g, 5min) and dump supernatant.

15 Re-suspend the nuclei with 1ml NSB. Pellet the nuclei (500g, 5min) and remove supernatant as much as possible with pipetting.

16 Re-suspend the nuclei with 500ul NSB. Split the nuclei into five tubes with 100ul each. Flash freeze the nuclei in liquid nitrogen and store the nuclei in liquid nitrogen or -80C freezer(stop point).

Nuclei permeabilization before reverse transcription (1 hour for 6 samples)

17 Preparation before the experiment: Cool the centrifuge to 4C.
Prepare nuclei suspension buffer (NSB): for 25 samples, combine 40ml nuclei buffer, 400ul SUPERase In RNase Inhibitor (20 U/μL, Ambion), and 400ul BSA (20 mg/ml, NEB). Store the mixture on ice.
Prepare nuclei buffer with 1% BSA (NBB): 100ml nuclei buffer and 1ml BSA (20 mg/ml, NEB). Store the buffer on ice.

18 Thaw paraformaldehyde (PFA) fixed nuclei (100ul flash-frozen in liquid nitrogen) in 37C water bath.

19 Pellet the nuclei at 500g for 5min (4C) and remove the supernatant.

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20 Add 100ul NSB to resuspend the nuclei.
For each sample, add mixture containing 390ul NSB and 10ul 10% Triton X-100.

21 Incubate the mix on ice for 3min.

22 Pellet the nuclei at 500g for 5min (4C) and remove the supernatant; Resuspend the nuclei in 400ul NSB;

23 Perform sonication in the Bioruptor Sonication device (low power mode, 12s).

24 Filter the nuclei through theFlowmi cell strainer.

25 Pellet the nuclei at 500g for 5min (4C) and remove the supernatant;

26 Resuspend the nuclei in 100ul NSB and count the nuclei concentration with a hemocytometer.

Reverse transcription (~ 2 hours for 6 samples)

27 For each well of 4 x 96 well plates, add 80,000 nuclei in 22ul nuclei buffer (with wide bore tips); 2ul 10mM dNTP; and 2ul indexed oligo-dT primer (100uM);

28 Incubate the plates at 55 °C for 5 min. Immediately place the plates on ice.

29 Prepare the reverse transcription reaction mix by combining:
5X Superscript IV First-Strand Buffer: 3280ul
100mM DTT: 820ul
SuperScript IV reverse transcriptase: 820ul
RNaseOUT Recombinant Ribonuclease Inhibitor: 820ul
Mix well and distribute 14ul to each well.

30 Start the RT reaction with the following program: 4°C 2 minutes, 10°C 2 minutes, 20°C 2 minutes, 30°C 2 minutes, 40°C 2 minutes, 50°C 2 minutes and 55°C 15 minutes.

31 After the reaction, add 60ul ice-cold NBB into each well with a multichannel pipette.

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32 Pool the nuclei from all wells (with wide bore tip).

33 Pellet the nuclei (500g, 10min), and remove the supernatant.

Ligation (~ 2 hours)

34 Resuspend the cells in 4.3ml NSB, and distribute 10ul into each well of 4 x 96well plates.

35 Add 8ul indexed ligation oligos (100 uM) into each well with a multichannel pipette.

36 Prepare ligation master mix by combining: Quick ligase buffer: 8.6ml; Quick ligase: 860ul; Mix well and distribute 22ul into each well with gently pipetting up and down five times (with wide bore tip).

37 Ligation at 25C for 10min.

38 add 60ul NBB into each well and pool the nuclei from all wells (with wide bore tips).

39 Add another 40ml NBB to the nuclei mix. Pellet the nuclei (600g, 10min) and dump the supernatant.

40 Resuspend the cells in 5ml NBB. Pellet the nuclei (600g, 10min) and dump the supernatant.

41 Resuspend cells in 4ml NBB. Filter the nuclei with the flowmi cell strainer (check the nuclei under the microscope and repeat the filtering step again if there are still a lot of doublets).

42 Count the nuclei concentration with a hemacytometer.

43 Distribute the diluted nuclei (in NBB) into several 96 well plates with 2500 to 4000 nuclei per well (5ul total volume).

44 Briefly spin the plates. The plates can be frozen in -80C for at least 1 month (stop point).

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Second strand synthesis (~ 3 hours)

45 Thaw one plate (with nuclei distributed per well) in room temperature.

46 Prepare second strand synthesis mix: Combine 330ul elution buffer, 146.7ul second strand synthesis buffer, and 73.3ul second strand synthesis enzyme and mix well; Distribute 5ul reaction mix per well and briefly centrifuge the plate.

47 Perform second strand synthesis at 16C for 3 hours.

Tagmentation (~10min)

48 Prepare the tagmentation mix by combining 1.1ml tagmentation buffer, 2.75ul N7 adaptor loaded Tn5 and mix well. Briefly centrifuge the plate and add a 10ul reaction mix into each well.

49 perform tagmentation at 55C at 5min;

50 Add 20ul DNA binding buffer per well and incubate the reaction mix at room temperature for 5min.

Ampure beads purification (~1 hour)

51 Prepare USER mix: combine 880ul nuclease-free water, 110ul 10X USER buffer, 110ul USER enzyme (NEB) and mix well.

52 For each well, add 40ul Ampure XP beads and mix well. Incubate the mixture at room temperature for 5min and briefly centrifuge the plate.

53 Put the plate on the magnetic stand for 5min and remove the supernatant.

54 Wash each well with 100ul 80% ethanol twice.

55 After the second wash, carefully remove all supernatant from each well and dry the plate at room temperature for 3min.

56 For each well, add 10ul USER reaction mix and seal the plate. Vortex the plate to mix the beads with the USER reaction mix. Briefly centrifuge and incubate the plate at 37C for 15min.

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57 Briefly centrifuge the plate. Add 7ul elution buffer to each well and mix well.

58 Briefly centrifuge the plate and leave the plate on the magnetic stand for 3min.

59 Transfer the supernatant (16ul) to a new 96 well plate (on ice).

PCR reaction and library preparation (~2 hours)

60 Briefly spin the plate.

61 For each well, add 2ul P5 PCR primer (10uM), 2ul P7 primer (10uM) and 20ul NEBnext master mix for PCR reaction: 72°C for 5 min, 98°C for 30 sec, 12 to 15 cycles of (98°C for 10 sec, 66°C for 30 sec, 72°C for 30 sec) and a final 72°C for 5 min (stop point).

62 Pool all wells together, and take 1ml PCR product for a normal 0.7X Ampure XP beads purification. Elute the product in 100ul elution buffer.

63 Perform a second round of 0.8x Ampure XP beads purification.

64 Library concentrations were determined by Qubit (Invitrogen) and the libraries were visualized by electrophoresis on a 6% TBE-PAGE gel (example image shown below).

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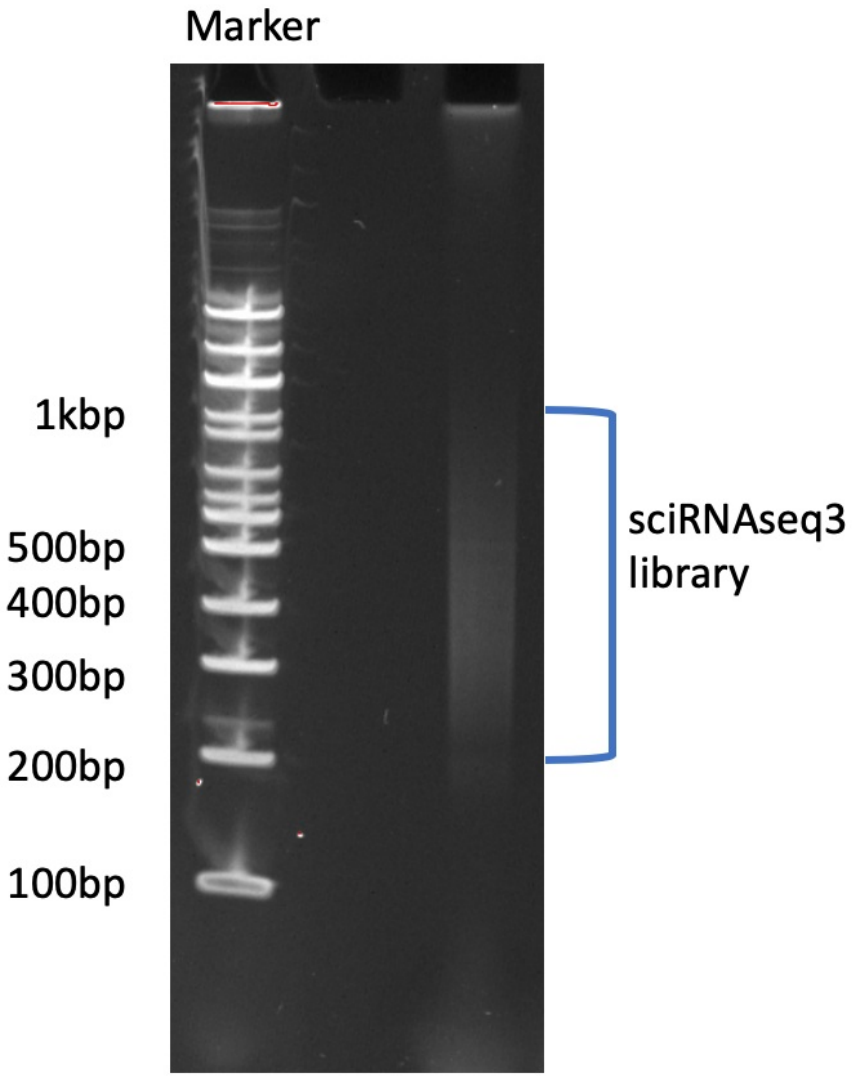

65 Library was sequenced on Novaseq platform (Read1: 34bp, Read2: 100bp, index 1: 10bp, index2: 10bp).

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