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scNT-seq2: single-cell metabolically labelled new RNA tagging sequencing for time-resolved analysis of gene expression in single cells

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We use this protocol and it's working

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Abstract

Single-cell metabolically labeled new RNA tagging sequencing (scNT-Seq) is a droplet microfluidics-based, time-resolved RNA-seq method for joint profiling of newly synthesized (“new”) and pre-existing (“old”) RNAs from the same cell by marking new transcripts with T-to-C substitutions via chemical conversion of 4-thiouridine (4sU) in new RNAs to cytidine analogs (Qiu et al., Nature Methods 17.10 (2020): 991-1001. PMID: 32868927). Here, we described the updated scNT-Seq2 method in which an optimized second-strand synthesis reaction is employed to increase the mapping rate and library complexity. This step-by-step protocol describes metabolic labeling, sample preparation, and scNT-Seq2 library preparation for Illumina sequencing.

Materials

	A	B	C
	Chemicals, Peptides, and Recombinant Proteins	Source	Identifier
	DPBS, no calcium, no magnesium	Invitrogen	14190136
	Barcoded beads	ChemGenes	MACOSKO-2011-10(V+)
	1M Tris-HCl, pH 8.0	Invitrogen	15568-025
	1 M Tris-HCl, pH 7.5	Invitrogen	15567-027
	0.5 M EDTA, pH 8.0	Invitrogen	15575-020
	NxGen RNase Inhibitor	Lucigen	30281-2
	Bovine Serum Albumin	Sigma-Aldrich	A8806-5G
	Ficoll PM-400	GE Healthcare/Fisher Scientific	45-001-745
	Sarkosyl	Sigma-Aldrich	L7414-50mL
	NaIO4	Sigma-Aldrich	7790-28-5
	sodium acetate	Invitrogen	AM9740
	Bst 3.0 DNA Polymerase	NEB	M0374
	Exonuclease I	NEB	M0293L
	TFEA	Sigma-Aldrich	269042-1G
	SPRISelect beads	Beckman Coulter	B23318
	DTT	Fermentas	R0862
	QX200 Droplet Generation Oil for EvaGreen	Bio-Rad	186-4006
	Perfluoro-1-octanol	Sigma-Aldrich	370533-25G
	dNTPs	Clontech	639125
	Critical Commercial Assays		
	Maxima H Minus Reverse Transcriptase	ThermoFisher	EP0753
	Nextera XT DNA sample preparation kit	Illumina	FC-131-1096
	KAPA HiFi hotstart readymix	KAPA Biosystems	KK2602
	Oligonucleotides		
	Template Switch Oligo	AAGCAGTGGTATCAACGCAGAGTGAATrGrGrG	
	TSO-PCR primer	AAGCAGTGGTATCAACGCAGAGT	
	Custom Read 1 Primer	GCCTGTCCGCGGAAGCAGTGGTATCAACGCAGAGTAC	
	TSO-N3G2N4B primer	AAGCAGTGGTATCAACGCAGAGTGA (N1:25252525)(N1)(N1)GG(N1)(N1)(N1)(N1)(N2: 00333433); N1 represents a mixture of A, C, G and T at a 25:25:25:25 ratio, N2 represents a mixture	

	A	B	C
		of A, C, G and T at a 0:33:34:33 ratio	
	P5-TSO hybrid primer	AATGATACGGCGACCACCGAGATC TACACGCCTGTCCGCGGAAGCAGT GGTATCAACGCAGAGT∗A∗C	

Metabolic labeling

1 Prepare a 1 M stock solution of 4-thiouridine (4sU) by dissolving the powder in DMSO.

> **Note**
>
> Note: the stock is stable for a few months at -20 C. Protect the solution from light.

2 For metabolic labeling, the medium was replaced with fresh medium supplemented with nontoxic concentrations of 4sU (i.e. 100 or 200 µM).

> **Note**
>
> If the labeling time is longer than 4 hours, regular exchange of fresh 4sU-containing media (i.e. every three hours) can enhance 4sU incorporation.

Prepare cell suspension

3 After metabolic labeling, cells were rinsed once with DPBS.

4 Add 0.5 mL Accutase to each well of the 6-well plate and incubate at 37 C for about 5 min.

5 Add 1 mL culture medium to neutralize the Accutase.

6 Collect cell suspension in the 15 mL tube, spin down cells at 350 g, 3min.

7 Resuspend the cell pellet with 1 mL of DPBS (containing 0.01% BSA + 0.5%RNase-Inhibitor).

8 Count the cell number with Countess II.

9 Dilute the cell with DPBS (containing 0.01% BSA + 0.5%RNase-Inhibitor) to 100 cells/ µL (1 X 10^5 cells/mL).

Cell and beads co-encapsulation

10 Prepare lysis buffer:

	A	B	C
	Reagents	Vol.(µL)	Final Concentration
	H2O	400	
	20% Ficoll PM-400	300	6%
	20% Sarkosyl	10	0.2%
	0.5 M EDTA	40	20 mM
	1.0 M Tris-HCl, pH 7.5	200	200 mM
	1.0 M DTT (add freshly)	50	50 mM

Lysis buffer master mix

11 Prepare barcoded beads:
Wash beads once with 30 mL of 100% ethanol and twice with 30 mL of TE-TW (10 mM Tris-HCl pH 8.0, 1 mM EDTA and 0.01% Tween-20). Pass the beads through a 100 µm cell strainer and count the number of beads. Resuspend the beads at 120 beads/µL concentration in 1.5 mL lysis buffer for each run (to profile 1,000~2,000 cells). Transfer 1.5 mL of bead suspension into a 3 mL Luer lock syringe. Make one more mL of lysis buffer to wash the syringe and magnetic disc.

12 Draw up 7 mL of droplet generation oil (Bio-Rad) into a 10-mL Luer-lock syringe.

13 Connect 3 syringes (containing cells, beads, and oil, respectively) to the Aquapel-coated PDMS Microfluidic device (µFluidix) with the following flow rate setting:

	A	B
	Syringe Content	Flow Rate (µL/hr)
	Oil	15000
	Cells	4000
	Beads	4000

14 Start the run in the following order: cells → beads → oil.

15 When the flow of droplets stabilizes, collect ~20 µL of aqueous flow to examine the droplet quality. Check whether the droplet size is uniform and estimate the percentage of bead doublets (the doublet rate should be less than 5%).

16 Once confirming the droplet quality, collect 1.2-1.3 mL of droplets into a 50 mL conical tube (target 1,000~2,000 cells for each sample).

Droplet breakage

17 Remove the oil layer from the bottom of the 50 mL tube.

18 Add 30 mL of room temperature 6X SSC into the tube.

19 Add 1 mL of Perfluorooctanol (PFO) into the tube in a fume hood. Shake by hand to break the droplets (3-4 forceful vertical shakes, the shakes should be long distance -- from head to leg) Spin at 1,000x g for 1 min.

20 Carefully remove the supernatant on top and then add 25 mL of 6X SSC to kick up the beads into the solution. Wait a few seconds to allow the majority of the oil to sink to the bottom. Transfer the supernatant to a new 50 mL tube.

21 Add 25 mL of 6X SSC to kick up the beads into thesolution again. Transfer and combine the supernatant.

22 Spin at 1,000x g for 1 min to pellet the beads.

23 The beads are now pelleted to the bottom of the tube. Carefully remove all but ~1 mL of liquid. Resuspend the beads with the remaining liquid and transfer them to a 1.5 mL Low Binding tube.

24 Spin at 1,000x g for 1 min. Remove the supernatant. Wash beads once with 1 mL of 6X SSC.

Chemical conversion of mRNAs on beads

25 Wash beads once with 450 µL reaction buffer (without TFEA and $NaIO_4$).

26 Incubate beads in 474 µL Reaction-mix + 26 µL $NaIO_4$ at 45 C for 1 hr with rotation.

	A	B
	Reagents	Volume (µL)
	3 M sodium acetate (pH 5.2)	8
	0.5 M EDTA (pH 8.0)	2
	H2O	214
	TFEA	13
	Sub-total	237
	192 mM NaIO4	13

Master mix for chemical conversion

27 Wash the beads once with 0.7 mL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5).

28 Incubate the beads in 0.7 mL Reducing Buffer at 37 C for 30 min with rotation. Add 2% RNase inhibitor (20 µL/1 mL) to the reducing buffer before use.

	A	B
	Reagents	For 1mL (µL)
	1 M Tris-HCl (pH7.5)	10
	1M DTT	10
	5M NaCl	20
	0.5M EDTA	2
	RNase Inhibitor	20
	DEPC-H2O	958

Reducing Buffer

Reverse transcription

29 Prepare RT mix:

	A	B
	Reagents	Vol. (µL)
	H2O	80
	Maxima 5X RT buffer	40

	A	B
	20% Ficoll PM-400	40
	10 mM dNTPs	20
	100 µM Template Switch Oligo	5
	RNase Inhibitor	5
	Maxima H Minus Reverse Transcriptase	10

30 Wash the beads once with 1 mL Tris-HCl buffer (10 mM, pH 7.5).

31 Wash the beads once with 0.3 mL 2X RT buffer.

32 Add 220 µL of RT mix to the beads.

33 Incubate beads at room temperature for 30 min with rotation, then 120 min at 42 C with rotation.

34 Wash beads once with 1 mL TE-SDS (10 mM Tris-HCl pH 8.0, 1 mM EDTA, and 0.5% SDS), twice with 1 mL TE-TW, then store the beads at 4°C over-night (in TE-TW buffer). [This is a Stop point. Store at 4C overnight (in TE-Tw buffer).]

Exonuclease I treatment

35 Prepare Exonuclease mix (makes 200 µL):

	A	B
	Reagents	Vol. (µL)
	10X Exonuclease I buffer	20
	H2O	170
	Exonuclease I	10

36 Wash beads once with 1 mL 10mM Tris-HCl pH 8.0, re-suspend in 190 µL of exonuclease mix.

37 *Incubate beads at 37 C for 45 min with rotation.*

38 Wash beads once with 1 mL TE-SDS, and twice with 1 mL TE-TW. [This is a Stop point. Store at 4C overnight (in TE-Tw buffer).]

Run 1st round of TSO-PCR using 6,000-beads as templates

39 Wash beads once with 1 mL H_2O. Spin at 1,000x g for 1 min.

40 Remove supernatant and re-suspend the beads with 1 mL of H2O. Quickly transfer 2 µL of beads into a well of a 96-well plate (containing 198 µL of H2O) and count the number of beads. Repeat bead counting three times and take the average.

41 Transfer an aliquot of 6,000 beads (corresponding to ~100 cells) into a PCR tube. Spin down and remove the supernatant, then re-suspend the beads with 50 µL PCR mix:

	A	B
	Reagents	Vol. (µL)
	KAPA HiFi HS Readymix	25
	100 µM TSO-PCR primer	0.4
	H2O	24.6

TSO-PCR master mix

42 Run 1st round of TSO-PCR. PCR program:
Very important: run 1st round of TSO-PCR to determine the exact number of amplification cycles. Over-amplification of the cDNA library will lead to fewer detected nuclei in the end).

95 C for 3 minutes
4 cycles of:
98 C for 20 seconds
65 C for 45 s
72C for 3 min
9 cycles of:
98 C for 20 s
67 C for 20 s
72 C for 3 min
Then:
72 C for 5 min
4 C forever

43 Purify PCR products once with 0.7X (35 µL) SPRI beads once and elute in 15 µL Elution buffer.

44 Measure the concentration of PCR products by Qubit.

45 Perform real-time PCR to determine the additional number of PCR cycles needed for optimal cDNA amplification.

	A	B
	Reagents	Vol. (µL)
	Purified cDNA	1
	25 µM TSO-PCR primer	0.2
	2X KAPA FAST qPCR Readymix	5
	H2O	3.8

Real-time PCR master mix

46 Run real-time PCR with the following program, and determine the optimal PCR cycle number
95 C for 3 min
25 cycles of:
95 C for 15 s
63 C for 30 s
72 C for 30 s

Second strand synthesis

47 Prepare second strand synthesis mix:

	A	B
	Reagents	Vol. (µL)
	10X Isothermal Amplification Buffer II	10
	20% Ficoll PM-400	20
	10 mM dNTPs	14
	Bst 3.0 DNA Polymerase (NEB)	5
	TSO-N3G2N4B primer (100 µM)	10
	MgSO4 (100 mM)	6
	H2O	35

Second strand synthesis master mix

48 After aspiration of TE-TW buffer, resuspend beads in 500 µL 0.1 M NaOH.

49 Incubate beads at room temperature for 5 min with rotation.

50 Add 500 µL 0.2M Tris-HCl (pH 7.5) to neutralize the solution.

51 Wash beads once with TE-TW and once with 10 mM Tris-HCl (pH 8.0).

52 Add 200 µL second strand synthesis mix to the beads. [Add Bst3 enzyme right before the reaction.]

53 Incubate beads at 15 min at 60 C with rotation.

54 Wash beads once with 1 mL TE-SDS, and twice with 1 mL TE-TW and twice with H2O.

Large-scale TSO-PCR

55 After determining the optimal PCR cycle number (usually an additional 0-4 cycles), perform large-scale TSO-PCR with the remaining beads. Wash the remaining beads twice with 1 mL H_2O. Apportion 6,000 beads for each PCR reaction. Spin down and remove the supernatant, then resuspend the beads with 50 µL PCR mix. PCR program:

95 C for 3 min
4 cycles of:
98 C for 20 s
65 C for 45 s
72 C for 3 min
X plus additional cycles of:
98 C for 20 s
67 C for 20 s
72 C for 3 min
Then:

72 C for 5 min
4 C forever

56 Combine the PCR product for a given sample into a 1.5 mL Low Binding tube and purify **twice** with SPRI-select beads (0.6X Volume for the 1st & 0.7X Volume for the 2nd purification).

57 Elute the cDNA with 40 µL Low-EDTA TE.

58 Quantify the cDNA library by Qubit and run the bioanalyzer to check the average fragment size of the purified cDNA library (the expected average size of cDNA library is 800-1,500 bp).

Tagmentation (Nextera® XT DNA Sample Preparation kit)

59 Preheat the thermocycler to 55 C. For each sample, take out 1 ng of purified cDNA with H_2O in a total volume of 5 µL to a PCR tube.

60 Add 10 µL of Nextra TD buffer and 5 µL of Amplicon Tagmentation enzyme to each reaction. Mix by pipetting ~5 times.

61 Incubate at 55 C for 5 min.

62 Add 5 µL of Neutralization Buffer to each reaction. Mix by pipetting ~5 times. Spin down and incubate at room temperature for 5 min.

63 Add to each PCR tube in the following order:

	A	B
	Reagents	Vol. (µL)
	Nextra PCR mix	15
	2 µM P5-TSO hybrid primer	5
	2 µM Nextera N70X oligo	5

64 *PCR program:*
95 C for 30 s
12 cycles of:
95 C for 10 s
55 C for 30 s
72 C for 30 s

Then:
72 C for 5 min
4 C forever

65 Purify PCR product twice with 0.6X SPRI beads. Elute the cDNA in 12 µL H_2O.

66 Quantify the concentration of cDNA library by Qubit and check the average fragment size of the purified cDNA library by Bioanalyzer (the expected fragment size is 500-700 bp).

Sequencing

67 Dilute the library to 2 nM and pool the libraries according to the estimated cell numbers (100 cells/6000 beads).

Protocol references

1. Hu, Peng, et al. "Dissecting cell-type composition and activity-dependent transcriptional state in mammalian brains by massively parallel single-nucleus RNA-seq." *Molecular cell* 68.5 (2017): 1006-1015.

2. Qiu, Qi, et al. "Massively parallel and time-resolved RNA sequencing in single cells with scNT-seq." *Nature methods* 17.10 (2020): 991-1001.