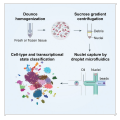

sNucDrop-seq Protocol

Version 2

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ABSTRACT

Massively parallel single-cell RNA sequencing can precisely resolve cellular diversity in a high-throughput manner at low cost, but unbiased isolation of intact single cells from complex tissues such as adult mammalian brains is challenging. Here, we integrate sucrose-gradient-assisted purification of nuclei with droplet microfluidics to develop a highly scalable single-nucleus RNA-seq approach (sNucDrop-seq), which is free of enzymatic dissociation and nucleus sorting. By profiling ~18,000 nuclei isolated from cortical tissues of adult mice, we demonstrate that sNucDrop-seq not only accurately reveals neuronal and non-neuronal subtype composition with high sensitivity but also enables in-depth analysis of transient transcriptional states driven by neuronal activity, at single-cell resolution,*in vivo.*

EXTERNAL LINK

https://www.cell.com/molecular-cell/fulltext/S1097-2765(17)30876-6?innerTabgraphical_S1097276517308766=#secsectitle0035

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

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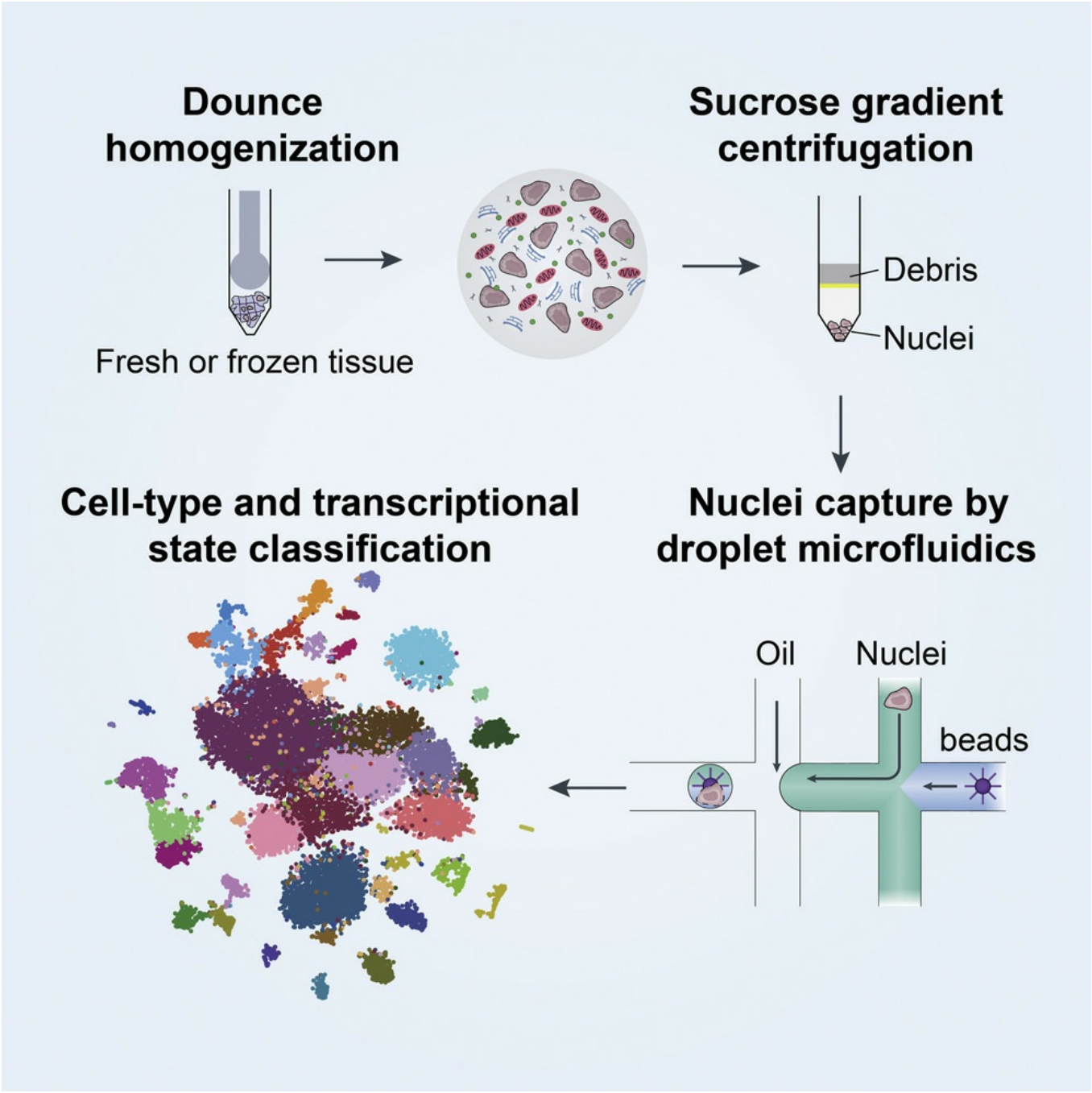
PROTOCOL STATUS

Working

We use this protocol in our group and it is working

GUIDELINES



MATERIALS TEXT

Chemicals, Peptides, and Recombinant Proteins	Source	Identifier
Dulbecco's Modified Eagle's Medium	Life Technologies	11965084
Fetal Bovine Serum	Life Technologies	26140079
L-glutamine	Life Technologies	25030081
0.05% Trypsin	Life Technologies	25300054
Matrigel matrix	Corning	354230
DMEM/F12	Life Technologies	11320033
TeSR-E8 Medium	Stem Cell Technologies	05940
DPBS, no calcium, no magnesium	Invitrogen	14190136
Sucrose	Sigma-Aldrich	S0389-1KG

1M Tris-HCl, pH 8.0	Invitrogen	15568-025
$MgAc_2$	Sigma-Aldrich	M5661-50G
cOmplete™, EDTA-free Protease Inhibitor Cocktail	Roche	11873580001
$CaCl_2$	Sigma-Aldrich	C1016-500G
Triton X-100	Sigma-Aldrich	T8787-100mL
0.5M 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
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
KAPA HiFi hotstart readymix	KAPA Biosystems	KK2602
Deposited Data		
Raw and analyzed data	This paper	GEO: GSE106678
Experimental Models: Cell Lines		
NIH3T3	ATCC	CRL-1658
H7 (female, human embryonic stem cells)	WiCell	WA07
Experimental Models: Organisms/Strains		
Mouse: C57BL/6J	Jackson Laboratory	000664
Oligonucleotides		
Template Switch Oligo: AAGCAGTGGTATCAACGC AGAGTGAATrGrGrG	Macosko et al., 2015	N/A
TSO-PCR primer: AAGCAGTGGTATCAACGCAGAGT	Macosko et al., 2015	N/A
P5-TSO hybrid primer: AATGATACGGCGACCACCG AGATCTACACGCCTGTCCGCGGAAGCAGTGG TAT CAACGCAGAGT*A*C	Macosko et al., 2015	N/A
Custom Read 1 Primer: GCCTGTCCGCGGAAGCA GTGGTATCAACGCAGAGTAC	Macosko et al., 2015	
Other		
Tube, Thinwall, Polypropylene, 38.5 mL, 25 x 89 mm (qty. 50)	Beckman Coulter	326823
Glass 15mL Dounce Tissue Grinder Set with Two Glass Pestles, Grinding Chamber O.D. x L: 22 x 94mm (Case of 2)	Wheaton	357544
SW 28 Ti Rotor, Swinging Bucket, Aluminum, 6 x 38.5 mL, 28,000 rpm, 141,000 x g	Beckman Coulter	342207
Barcoded Beads	ChemGenes	MACOSKO-2011-10
PDMS Microfluidic Device	µFluidix	Batch #9508
Syringe Pumps	KD Scientific	78-8100
40µm Sterile Cell Strainer	Fisher Scientific	22-363-547
Medical Grade Polyethylene Micro Tubing	Scientific Commodities	BB31695-PE/2
SPRISelect Beads	Beckman Coulter	B23318
75-cycle High Output v2 Kit	Illumina	FC-404-2005

10-micron carboxylated polystyrene beads	Bangs Labs	#PC06N-11355

Software and Algorithms	
Drop-seq_tools (v1.12)	http://mccarrolllab.com/dropseq/
STAR v2.5.2a	https://github.com/alexdobin/STAR
Seurat v1.4	http://satijalab.org/seurat/
Seurat v2.0	http://satijalab.org/seurat/
GSEA	http://software.broadinstitute.org/gsea/index.jsp
DBSCAN	https://cran.r-project.org/web/packages/dbscan/index.html
MISO	https://miso.readthedocs.io/en/fastmiso/
Random Forest	https://www.stat.berkeley.edu/~breiman/RandomForests/

Nuclei Isolation

1

Prepare sucrose cushion (50 mL):

Reagents	Vol.
2 M Sucrose	45 mL
H2O	4.45 mL
1 M Tris-HCl pH 8.0	500 µL
3 M MgAc2	50 µL
1 tablet protease inhibitor w/o EDTA	

NOTE

Cool down the ultracentrifuge to 4 °C before preparing buffers.

2

Prepare homogenization buffer (50 mL):

Reagents	Vol.
H2O	40.89 mL
2 M Sucrose	8 mL
0.5 M CaCl2	500 µL
1 M Tris-HCl pH 8.0	500 µL
3 M MgAc2	50 µL
100% Triton	50 µL
0.5 M EDTA	10 µL
1 tablet protease inhibitor w/o EDTA	

3 Add 14 mL of sucrose cushion to the bottom of a 1 x 3.5 in (25 x 98 mm) centrifuge tube (Beckman). Keep centrifuge tube on ice.

14 ml Sucrose cushion

4 Add 12 mL of homogenization buffer into the dounce tissue grinder, douncing 21 times with loose pestle then 7 more times using tight pestle to release the nuclei from adult mouse cortical tissues.

12 ml **Homogenization buffer**

NOTE

This step needs to be optimized for different brain regions and other adult tissues.

5 Carefully transfer the homogenate atop the sucrose cushion in the centrifuge tube. Add an additional 10 mL of the homogenization buffer atop the homogenate.

12 ml **Homogenization buffer**

NOTE

Add the solution into the centrifuge tube slowly.

6 Centrifuge at 25,000 rpm for 2 hours at 4 °C to pellet nuclei.

02:00:00 **Centrifugation**

4 °C

7 After centrifugation, carefully remove the centrifuge tube from rotor and keep the tubes on ice.

NOTE

An off-white circular pellet containing nuclei should be visible at the bottom of centrifuge tube.

8 Carefully remove the supernatant and add 1 mL chilled resuspension buffer (0.01% BSA in DPBS with RNase inhibitor). Incubate on ice for 20 min beforere suspending the pellet.

1 ml **Resuspension buffer**

00:20:00 **Incubation on ice**

9 Resuspend nuclei pellet and transfer the suspension into a 1.5-mL lobind tube (Eppendorf).

NOTE

Wash centrifuge tube with 0.5 mL chilled resuspension buffer and add to Eppendorf tube.

10 Spin down nuclei at 5,000 rpm for 15 minutes at 4 °C to pellet nuclei. Remove supernatant, and resuspend in 1.5 mL resuspension buffer.

00:15:00 **Centrifugation**

1.5 ml **Resuspension buffer**

NOTE

To remove any excess sucrose cushion from the buffer.

11 Filter nuclei twice with 40-µm cell strainer and count the number of nuclei. Dilute nuclei to 100 nuclei/µL with resuspension buffer(0.01% BSA in DPBS with RNase inhibitor). Transfer 2 mL of nuclei suspension into a 3-mL Luer-lock syringe.

NOTE
The following steps were adapted from the Drop-seq protocol (Macosko et al., 2015).

Nuclei and beads co-encapsulation

12 Prepare barcoded beads (ChemGenes):

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)

30 ml 100% Ethanol

30 ml TE-TW

13 Pass beads through a 100-µm cell strainer and count the number of beads.

14 Re-suspend beads at 120 beads/µL concentration in 1.5 mL lysis buffer for each sNucDrop-seq run. Transfer 1.5 mL of bead suspension into a 3-mL Luer lock syringe.

1.5 ml Lysis Buffer

15 Prepare lysis buffer (makes 1 mL):

Reagents	Vol. (µL)
H2O	400
20% Ficoll PM-400	300
20% Sarkosyl	10
0.5 M EDTA	40
1.0 M Tris-HCl, pH 7.5	200
1.0 M DTT	50

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

7 ml Droplet generation oil

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

Syringe Content	Flow Rate (µL/hr)
Oil	15,000
Nuclei	4,000
Beads	4,000

18 Start the run by pressing “start” on the pumps in the following order: nuclei→ beads → oil.

19 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%).

20 Once confirming the droplet quality, collect 1.0 mL of droplets into a 50-mL conical tube.

Droplet Breakage

21 Remove the oil layer from the bottom of the 50-mL tube.

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

30 ml 6X SSC

23 Add 1 mL of Perfluorooctanol (PFO) into the tube in a fume hood. Shake, rigorously,the tube 4 times to break the droplets. Spin at 1000Xg for 1 min.

1 ml PFO

00:01:00 Centrifugation

24 Carefully remove the supernatant on top and then add 20 mL of 6X SSC to to kick up the beads into 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.

20 ml 6X SSC

25 Add 20 mL of 6X SSC to kick up the beads into solution again. Transfer and combine the supernatant.

20 ml 6X SSC

26 Spin at 1000xg for 2 min to pellet the beads.

00:02:00 Centrifugation

NOTE

Make the RT master mix.

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

28 Spin 1000X g for 1min. Remove the supernatant. Wash beads twice with 1 mL of 6X SSC and then once with ~300 µL 2X RT buffer. Remove as much of the 2X RT buffer as you can without taking any beads.

00:01:00 Centrifugation

1 ml 6X SSC

300 µl 2X RT

Reverse Transcription

29 Prepare RT mix (makes 200 µL):

Reagents	Vol. (µL)
H2O	80
Maxima 5X RT buffer	40
20% Ficoll PM-400	40
10 mM dNTPs	20
100 µM Template Switch Oligo	5
RNase Inhibitor (Lucigen)	5
Maxima H Minus Reverse Transcriptase	10

30 Add 200 µL of RT mix to the beads.

200 µl RT Mix

31 Incubate beads at room temperature for 30 min with rotation, then 150 min at 42 °Cwith rotation.

00:30:00 Room Temperature

02:30:00 at 42 °C

32

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.

1 ml TE-SDS

1 ml TE-TW

NOTE

Beads can be stored at 4°C in TE-TW for at least one month.

Exonuclease I treatment

33 Prepare Exonuclease mix (makes 200 µL):

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

34 Wash beads once with 1 mL 10mM Tris-HCl pH 8.0, re-suspend in 200 µL of exonuclease mix.

1 ml 10mM Tris-HCl pH8.0

200 µl exonuclease mix

35 Incubate beads at 37°C for 45 min with rotation.

00:45:00 Incubation at 37°C

36 Wash beads once with 1 mL TE-SDS, twice with 1 mL TE-TW.

1 ml TE-SDS

1 ml TE-TW

cDNA Amplification

37 Wash beads once with 1 mL H_2O. Spin 1000X g for 1 min.

1 ml H2O

38 Remove supernatant and re-suspend the beads with 1 mL of H_2O.Quickly transfer 1 µLof beads into a well of 96-well plate (containing 50 µL of H_2O) and count the number of beads. Repeat bead counting three times and take the average.

1 ml H2O

NOTE

The expected bead concentration is 40-70 beads/µL for each run (~1 mL of droplets).

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

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

Store the remaining beads in TE-TW at 4°C.

NOTE

Run 1st round of TSO-PCR to determine the optimal number of PCR amplification cycle to avoid over-amplification.

40 Run 1st round of TSO-PCR.PCR program:

95 °C for 3 minutes (min)
4 cycles of:
98 °C for 20 seconds (s)
65 °C for 45 s
72°C 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

41 Purify PCR productstwice with0.6X (30 µL) SPRI beads. Elute in 10 µL H_2O.

30 µl SPRI beads

10 µl H2O

42 Measure the concentration of PCR products by Qubit.

NOTE
For mouse cortical nuclei, the expected concentrationis 100-600 pg/µL.

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

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

44 Run real-time PCR with the following program:

95 °C for 3 min
25 cycles of:
95 °C for 15 s
63 °C for 30 s
72°C for 30 s

45 After determining the optimal PCR cycle number, perform large-scale TSO-PCR on 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 re-suspend the beads with 50 µL PCR mix.

1 ml H2O

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
9 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

46 Combine the PCR product for a given sample into a 1.5-mL lobind tube and purify twice with 0.6X SPRI beads. Elute the cDNA in H_2O (in 1/5 the volume of the PCR product).

47 Quantify the cDNA library by Qubit (for mouse cortical nuclei, the expected concentrationis 800-2000 pg/µL) and run the bioanalyzer to check the average fragment size of the purified cDNA library (the expected average size of cDNA library is 1300-2000 bp).

Tagmentation

48 Preheat thermocycler to 55°C. For each sample, take out 550 pg of purified cDNA with H_2O in a total volume of 5 µL to a PCR tube.

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

10 µl Nextera

50 Incubate at 55 °C for 5 min.

00:05:00 Incubation

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

5 µl Neutralization Buffer

00:05:00 Incubation

52 Add to each PCR tube in the following order:

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

53 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

54 Purify PCR product twice with 0.6XSPRI beads. Elute the cDNA in 10µL H_2O.

10 µl H20

55 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 is500-700 bp).

Sequencing

56 Estimate the library concentration:
Conc. (nM) =

57 For sequencing on Illumina NextSeq 500 (75-cycle High Output v2 Kit), denature the library and dilute to 2.0 pM. Load diluted library to position 10. Dilute custom Read1 primer (0.3 µM) and load it to position 7. For sequencing multiple libraries in one flowcell, use following

sequencing specification: 20 bp (Read1; custom read1 primer), 8 bp (Index1), and 50-60 bp (Read2).