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Single Nucleus Drop-seq (snDrop-seq)

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1 Works for me dx.doi.org/10.17504/protocols.io.zmvf466

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Zhang Lab

ABSTRACT

The protocol presented here is a Drop-Seq protocol modified for single nuclei.

The original Drop-Seq protocol comes from the McCarroll Lab in the Department of Genetics, Harvard Medical School.
http://mccarrolllab.org/download/905/

EXTERNAL LINK

http://genome-tech.ucsd.edu/ZhangLab/

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Lake, B.B., Chen, S., Hoshi, M. et al. A single-nucleus RNA-sequencing pipeline to decipher the molecular anatomy and pathophysiology of human kidneys. Nat Commun 10, 2832 (2019). https://doi.org/10.1038/s41467-019-10861-2

MATERIALS

NAME	CATALOG #	VENDOR
DTT	D0632	Sigma Aldrich
15 mL Falcon tubes		
PCR tubes		
SSC	AM9765	Thermo Fisher Scientific
10x PBS	AM9624	Thermo Fisher Scientific
UltraPure Distilled Water	10977-015	Invitrogen - Thermo Fisher
TrypLE™ Express Enzyme	12604013	Thermo Fisher Scientific
TSO: AAGCAGTGGTATCAACGCAGAGTGAATrGrGrG	View	Integrated DNA Technologies
SMART PCR primer: AAGCAGTGGTATCAACGCAGAGT	View	Integrated DNA Technologies
New-P5-SMART PCR hybrid oligo: AATGATACGGCGACCACCGAGATCTACACGCCT GTCCGCGGAAGCAGTGGTATCAACGCAGAGT* A*C	View	Integrated DNA Technologies
Custom Read 1 primer: GCCTGTCCGCGGAAGCAGTGGTATCAACGCAG AGTAC	View	Integrated DNA Technologies
BSA	#A8806	Sigma Aldrich
Barcoded Bead SeqB: 5' –Bead–Linker-TTTTTTTAAGCAGTGGTATCAAC GCAGAGTACJJJJJJJJJJJJNNNNNNNN TTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-3'	View	Chemgenes
Inverted Microscope	Motic AE31	

Citation: Song Chen, Blue B. Lake, Sarah Urata, Kun Zhang (04/10/2020). Single Nucleus Drop-seq (snDrop-seq). https://dx.doi.org/10.17504/protocols.io.zmvf466

NAME	CATALOG #	VENDOR
Syringe Pumps (3)	Legato 100	
Magnetic Stirrer	710D2	VP Scientific
Magnetic Mixing Discs	772DP-N42-5-2	VP Scientific
3 mL syringes	BD #309657	BD Biosciences
10 mL syringes	BD 309695	BD Biosciences
Tubing	BB31695PE/2	Scientific Commodities
Luer lock 26-gage needles	305111	BD Biosciences
PDMS co-flow microfluidic droplet generation device	CAD file from McCarroll Lab	
100 micron cell strainers (for beads)	21008-950	VWR international Ltd
40 micron cell strainers (for cells)	21008-949	VWR international Ltd
Fuchs-Rosenthal hemocytometer	DHC-F01	INCYTO
Ficoll PM-400 20% in H2O	F5415-50ML	Sigma Aldrich
Nuclease-free H2O	AM9930	Life Technologies
Sarkosyl	L7414	Sigma Aldrich
EDTA 0.5 M	AM9260G	Life Technologies
Tris 2M pH 7.5	T2944	Sigma Aldrich
Perfluorooctanol	370533	Sigma Aldrich
30 um Uberstrainer	43-70030-03	pluriSelect
50 mL centrifuge tubes	734-1876	VWR International
Droplet Generation Oil	186-4006	Bio-rad Laboratories
Mineral Oil	M5904	Millipore Sigma
Tris 2M pH 8.0	T3069	Sigma Aldrich
10% SDS	S0288	Teknova
Tween-20	P9416	Sigma Aldrich
Maxima 5X RT Buffer	View	Thermo Fisher Scientific
Maxima™ H Minus Reverse Transcriptase	EP0753	Thermo Fisher Scientific
10 mM dNTPs	639125	Takarabio
RNase Inhibitor	30281-1	Lucigen
10x Exo I Buffer	View	Thermo Fisher Scientific
Exo I	FEREN0582	Thermo Fisher Scientific
Kapa HiFi Hotstart Readymix	KK2601	Kapa Biosystems
Agencourt AMPure XP beads	A63881	Beckman Coulter

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NAME	CATALOG #	VENDOR
Qubit 4 Fluorometer	Q33238	Thermo Fisher Scientific
Qubit dsDNA HS Assay Kit	Q32854	Thermo Fisher Scientific
Nextera XT DNA Library Preparation Kit	FC-131-1096	Illumina, Inc.
MiSeq v3 (150 cycle) Kit	MS-102-3001	Illumina, Inc.

STEPS MATERIALS

NAME	CATALOG #	VENDOR
Syringe Pumps (3)	Legato 100	
Inverted Microscope	Motic AE31	
PDMS co-flow microfluidic droplet generation device	CAD file from McCarroll Lab	
Tubing	BB31695PE/2	Scientific Commodities
15 mL Falcon tubes		
Barcoded bead, sequence: TTTTTTTAAGCAGTGGTATCAACGCAGAGTACJJJJJJJJJJJJ NNNNNNNNT(30); where J=split-pool oligo; N=random oligo	Macosko-2011- 10	Chemgenes
Ficoll PM-400 20% in H2O	F5415-50ML	Sigma Aldrich
Nuclease-free H2O	AM9930	Life Technologies
Sarkosyl	L7414	Sigma Aldrich
EDTA 0.5 M	AM9260G	Life Technologies
Tris 2M pH 7.5	T2944	Sigma Aldrich
DTT	D0632	Sigma Aldrich
BSA	#A8806	Sigma Aldrich
SSC	AM9765	Thermo Fisher Scientific
EDTA 0.5 M	AM9260G	Life Technologies
10% SDS	S0288	Teknova
EDTA 0.5 M	AM9260G	Life Technologies
Tween-20	P9416	Sigma Aldrich
Tris 2M pH 8.0	T3069	Sigma Aldrich
Syringe Pumps (3)	Legato 100	
Inverted Microscope	Motic AE31	
Magnetic Stirrer	710D2	VP Scientific
Barcoded bead, sequence: TTTTTTTAAGCAGTGGTATCAACGCAGAGTACJJJJJJJJJJJJ NNNNNNNNT(30); where J=split-pool oligo; N=random oligo	Macosko-2011- 10	Chemgenes
100 micron cell strainers (for beads)	21008-950	VWR international Ltd
Fuchs-Rosenthal hemocytometer	DHC-F01	INCYTO
10 mL syringes	BD 309695	BD Biosciences
3 mL syringes	BD #309657	BD Biosciences

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NAME	CATALOG #	VENDOR
Magnetic Mixing Discs	772DP-N42-5-2	VP Scientific
Tubing	BB31695PE/2	Scientific Commodities
Luer lock 26-gage needles	305111	BD Biosciences
50 mL centrifuge tubes	734-1876	VWR International
30 um Uberstrainer	43-70030-03	pluriSelect
Mineral Oil	M5904	Millipore Sigma
Perfluorooctanol	370533	Sigma Aldrich
Maxima 5X RT Buffer	View	Thermo Fisher Scientific
Nuclease-free H2O	AM9930	Life Technologies
Maxima 5X RT Buffer	View	Thermo Fisher Scientific
Ficoll PM-400 20% in H2O	F5415-50ML	Sigma Aldrich
10 mM dNTPs	639125	Takarabio
RNase Inhibitor	30281-1	Lucigen
TSO: AAGCAGTGGTATCAACGCAGAGTGAATrGrGrG	View	Integrated DNA Technologies
Maxima™ H Minus Reverse Transcriptase	EP0753	Thermo Fisher Scientific
10x Exo I Buffer	View	Thermo Fisher Scientific
Nuclease-free H2O	AM9930	Life Technologies
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Kapa HiFi Hotstart Readymix	KK2601	Kapa Biosystems
Agencourt AMPure XP beads	A63881	Beckman Coulter
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Nuclease-free H2O	AM9930	Life Technologies
New-P5-SMART PCR hybrid oligo: AATGATACGGCGACCACCGAGATCTACACGCCT GTCCGCGGAAGCAGTGGTATCAACGCAGAGT* A*C	View	Integrated DNA Technologies
Agencourt AMPure XP beads	A63881	Beckman Coulter
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NAME	CATALOG #	VENDOR
Agencourt AMPure XP beads	A63881	Beckman Coulter
Custom Read 1 primer: GCCTGTCCGCGGAAGCAGTGGTATCAACGCAG AGTAC	View	Integrated DNA Technologies
MiSeq v3 (150 cycle) Kit	MS-102-3001	Illumina, Inc.
10x PBS	AM9624	Thermo Fisher Scientific
UltraPure Distilled Water	10977-015	Invitrogen - Thermo Fisher

Prepare Buffers and Solutions

1 **BSA**

Make a 10% stock solution using BSA powder

BSA
by Sigma Aldrich
Catalog #: #A8806

2 **1X PBS**

Make a 1X stock solution using by diluting 10X PBS into nuclease-free distilled water

10x PBS
by Thermo Fisher Scientific
Catalog #: AM9624

UltraPure Distilled Water
by Invitrogen - Thermo Fisher
Catalog #: 10977-015

Citation: Song Chen, Blue B. Lake, Sarah Urata, Kun Zhang (04/10/2020). Single Nucleus Drop-seq (snDrop-seq). https://dx.doi.org/10.17504/protocols.io.zmvf466

3 **Cell Loading Buffer** (makes **1 ml**)

Prepare enough for the number of samples (**1 ml per sample**) that will be processed in one experiment

300 µl 20% Ficoll PM-400

Ficoll PM-400 20% in H2O
by Sigma Aldrich
Catalog #: F5415-50ML

700 µl 1X PBS

4 **PBS-BSA**: make this fresh before each experiment
- Cell Loading Buffer
- 0.01% BSA (use the 10% stock)

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5 **Lysis Buffer** (makes **1.2 ml**)

Prepare enough for the number of samples (**1 ml per sample**) that will be processed in one experiment

960 µl H2O

Nuclease-free H2O
by Life Technologies
Catalog #: AM9930

12 µl 20% Sarkosyl

Sarkosyl
by Sigma Aldrich
Catalog #: L7414

48 µl 0.5 M EDTA

EDTA 0.5 M
by Life Technologies
Catalog #: AM9260G

120 µl 2M Tris, pH 7.5

Tris 2M pH 7.5
by Sigma Aldrich
Catalog #: T2944

60 µl 1M DTT

Add this just prior to starting each DropSeq experiment

DTT
by Sigma Aldrich
Catalog #: D0632

Citation: Song Chen, Blue B. Lake, Sarah Urata, Kun Zhang (04/10/2020). Single Nucleus Drop-seq (snDrop-seq). https://dx.doi.org/10.17504/protocols.io.zmvf466

6 **6X SSC**
Make 6X SSC working stock from 20X SSC

SSC
by Thermo Fisher Scientific
Catalog #: AM9765

7 **10 mM Tris pH 8.0**
Make 10 mM Tris pH 8.0 working stock from 2M Tris pH 8.0

Tris 2M pH 8.0
by Sigma Aldrich
Catalog #: T3069

8 **TE-SDS** (make **50 ml**)
- 10 mM Tris pH 8.0 + 1 mM EDTA
- 0.5% SDS

EDTA 0.5 M
by Life Technologies
Catalog #: AM9260G

10% SDS
by Teknova
Catalog #: S0288

Citation: Song Chen, Blue B. Lake, Sarah Urata, Kun Zhang (04/10/2020). Single Nucleus Drop-seq (snDrop-seq). https://dx.doi.org/10.17504/protocols.io.zmvf466

9 **TE-TW** (make **500 ml**)

- 10 mM Tris pH 8.0 + 1 mM EDTA
- 0.01% Tween-20

EDTA 0.5 M
by Life Technologies
Catalog #: AM9260G

Tween-20
by Sigma Aldrich
Catalog #: P9416

10 **RT mix** (makes **200 µl**)

75 µl H2O

Nuclease-free H2O
by Life Technologies
Catalog #: AM9930

40 µl Maxima 5x RT Buffer

Maxima 5X RT Buffer
by Thermo Fisher Scientific
View

40 µl 20% Ficoll PM-400

Ficoll PM-400 20% in H2O
by Sigma Aldrich
Catalog #: F5415-50ML

20 µl 10 mM dNTPs

10 mM dNTPs
by Takarabio
Catalog #: 639125

5 µl RNase Inhibitor

Citation: Song Chen, Blue B. Lake, Sarah Urata, Kun Zhang (04/10/2020). Single Nucleus Drop-seq (snDrop-seq). https://dx.doi.org/10.17504/protocols.io.zmvf466

RNase Inhibitor
by Lucigen
Catalog #: 30281-1

10 µl 50 uM Template Switch Oligo

TSO:
AAGCAGTGGTATCAACGCAGAGTGAA
TrGrGrG
by Integrated DNA Technologies
View

10 µl Maxima H- RTase

Add after beginning breakage portion of the protocol (Step 27).

Maxima™ H Minus Reverse Transcriptase
by Thermo Fisher Scientific
Catalog #: EP0753

11 **Exonuclease Mix** (makes **200 µl**)

20 µl 10x Exo I Buffer

10x Exo I Buffer
by Thermo Fisher Scientific
View

170 µl H2O

Nuclease-free H2O
by Life Technologies
Catalog #: AM9930

10 µl Exo I

Exo I
by Thermo Fisher Scientific
Catalog #: FEREN0582

Citation: Song Chen, Blue B. Lake, Sarah Urata, Kun Zhang (04/10/2020). Single Nucleus Drop-seq (snDrop-seq). https://dx.doi.org/10.17504/protocols.io.zmvf466

Droplet Formation

12 Preparing the Barcoded Beads from Dry Resin

Barcoded bead, sequence: TTTTTTTAAGCAGTGGTATCAACGCAG AGTACJJJJJJJJJJJJ NNNNNNNNT(30); where J=split-pool oligo; N=random oligo
by Chemgenes
Catalog #: Macosko-2011- 10

The beads will arrive as a dry resin. To prepare the beads for an experiment from the dry resin, follow these steps:

1. Wash the resin with **30 ml ethanol**
2. Wash twice with **30 ml TE-TW**
3. Resuspend in **20 ml TE-TW**
4. Pass through 100 micron strainer
5. Count the beads using a Fuchs-Rosenthal hemocyctometer
6. Store the counted beads at **4 °C**

To pellet the beads when washing, centrifuge at 1000xg for **00:01:00**

Do NOT vortex the barcoded beads. Vortexing will shear the beads and cause damage. To mix, simply invert tube gently a couple of times until mixed.

100 micron cell strainers (for beads)
by VWR international Ltd
Catalog #: 21008-950

Fuchs-Rosenthal hemocytometer
by INCYTO
Catalog #: DHC-F01

13 Arrangement of components

Citation: Song Chen, Blue B. Lake, Sarah Urata, Kun Zhang (04/10/2020). Single Nucleus Drop-seq (snDrop-seq). https://dx.doi.org/10.17504/protocols.io.zmvf466

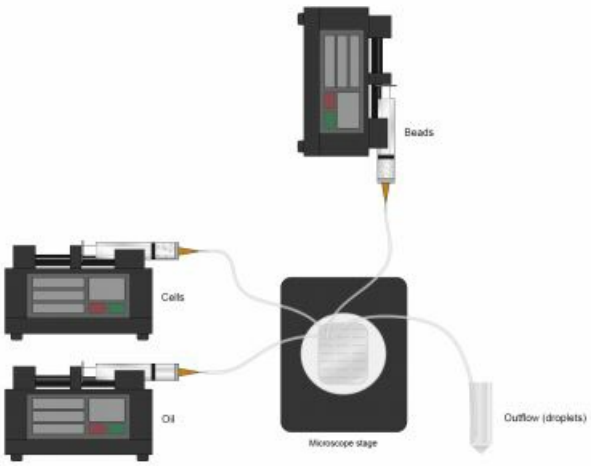
Syringe Pumps (3)
Catalog #: Legato 100

Inverted Microscope
Catalog #: Motic AE31

Magnetic Stirrer
by VP Scientific
Catalog #: 710D2

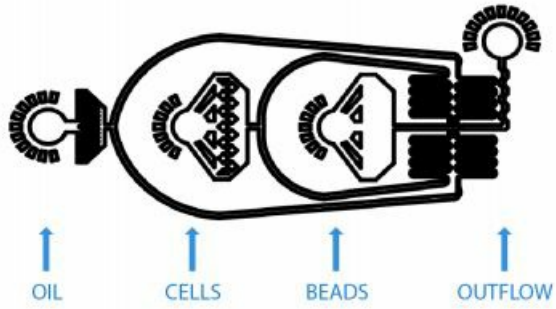
Citation: Song Chen, Blue B. Lake, Sarah Urata, Kun Zhang (04/10/2020). Single Nucleus Drop-seq (snDrop-seq). https://dx.doi.org/10.17504/protocols.io.zmvf466

Arrangement of components



Attachment of tubing into the device

Tubing is attached by pressing the tip of the tube into the circular punched holes.



Set up the syringe pumps next to the inverted microscope. Note: arrange the bead pump so that the syringe is pointing downward ratherly than horizontally. Place the magnet stirrer close to the barrel of the bead syringe.

Source: http://mccarrolllab.org/download/905/

14 Prepare Pump System

Syringe Pumps (3)
Catalog #: Legato 100

1. Power on the syringe pumps (switches located on the back left of the pump)
2. Set up correct flow rates using the screen on the pump
   - Oil: 15,000 ul/hr
   - Cells: 4,000 ul/hr
   - Beads: 4,000 ul/hr

Citation: Song Chen, Blue B. Lake, Sarah Urata, Kun Zhang (04/10/2020). Single Nucleus Drop-seq (snDrop-seq). https://dx.doi.org/10.17504/protocols.io.zmvf466

15 Prepare microfluidic cell

Inverted Microscope
Catalog #: Motic AE31

PDMS co-flow microfluidic droplet generation device
Catalog #: CAD file from McCarroll Lab

Tubing
by Scientific Commodities
Catalog #: BB31695PE/2

15 mL Falcon tubes

1. Cut and remove protective plastic top layer for one device on the chip
2. Cut the outflow tubing to proper length and connect it to the device so that the collection from the outflow can be safely collected
3. Use clean 15 mL tubes to catch the droplets
4. Use a waste container to catch waste

16 Prepare the beads

Barcoded bead, sequence: TTTTTTTAAGCAGTGGTATCAACGCAG AGTACJJJJJJJJJJJJ NNNNNNNNT(30); where J=split-pool oligo; N=random oligo
by Chemgenes
Catalog #: Macosko-2011- 10

1. Take an aliquot of beads (final concentration: 120,000 beads/mL)
2. Spin down in a tabletop centrifuge
3. Remove the TE-TW storage buffer
4. Wash with DTT minus lysis buffer
5. Resuspend in Lysis buffer

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17 Prepare nuclei according to the protocol "Isolation of single nuclei from solid tissues" steps 1-14.
dx.doi.org/10.17504/protocols.io.ufketkw

Dilute nuclei to 100 cells/ul. Use PBS-BSA for final dilution

Citation: Song Chen, Blue B. Lake, Sarah Urata, Kun Zhang (04/10/2020). Single Nucleus Drop-seq (snDrop-seq). https://dx.doi.org/10.17504/protocols.io.zmvf466

18 Prepare syringes and lines for microfluidic device

Prepare enough syringes and lines for all samples at once (4-6 samples per experiment)

1. Oil syringe: 10 ml
2. Cell/Sample syringes: 3 ml
3. Bead syringes: 3 ml with magnetic mixing disc
4. Measure out tubing for the lines; make sure they are long enough to reach the microfluidic device from where the syringes will sit on the pumps
5. For each line cut one blunt end (for blunt end needle) and one angled end (insert into the device)
6. UV all tubing and needles

10 mL syringes
by BD Biosciences
Catalog #: BD 309695

3 mL syringes
by BD Biosciences
Catalog #: BD #309657

Magnetic Mixing Discs
by VP Scientific
Catalog #: 772DP-N42-5-2

Tubing
by Scientific Commodities
Catalog #: BB31695PE/2

Luer lock 26-gage needles
by BD Biosciences
Catalog #: 305111

Citation: Song Chen, Blue B. Lake, Sarah Urata, Kun Zhang (04/10/2020). Single Nucleus Drop-seq (snDrop-seq). https://dx.doi.org/10.17504/protocols.io.zmvf466

19 Load Syringes

To load syringe, firmly press the tip of a 1000 ul pipette into the head of the syringe and slowly pull back on the plunger to draw in the solution. Pressing the tip in firmly helps reduce the introduction of bubbles. While holding the syringe in a vertical orientation, gently push out the air bubbles. Affix a 26G needle and tubing.

Before droplet generation, coat connecting tubing and syringes with 1% BSA to prevent non-specific binding of nuclei to the surface, and then rinse with PBS

1. Flush the tubing with corresponding buffer; keep syringes vertical when flushing
2. Use **1 ml cell buffer** to flush syringe/tube for each sample
3. Use **1 ml DTT minus lysis buffer** to flush syring/tube for each bead setup
4. Load the syringes; keep syringes vertical when loading
5. Load cells/nuclei into cell syringes, making sure there are no air bubbles
6. Load beads into bead syringes, making sure there are no air bubbles
7. After beads are loaded in the syringe, keep horizontal to prevent clogging

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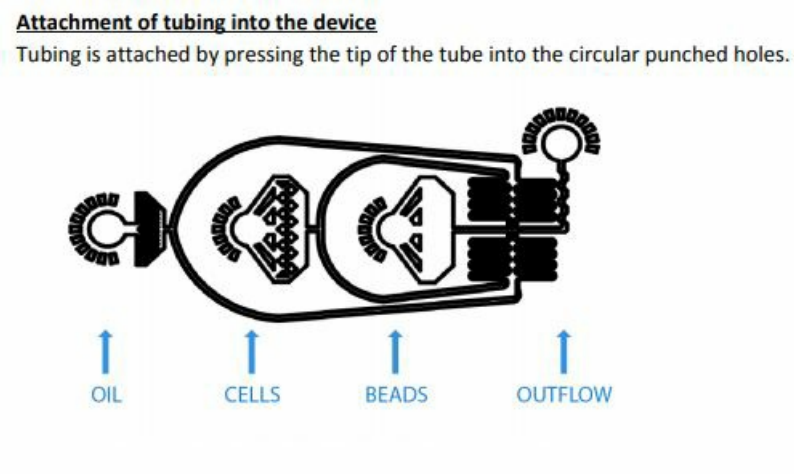
20 System Assembly

1. Place oil syringe in pump and secure; using the screen, hold the free tube over the waste collection, then slowly move the pusher to the plunger until oil drips out
2. Push the angled end of the tubing into the device
3. Place cell/sample syringe in pump and secure; using the screen, hold the free tube over the wase collection, then slowly move the pusher to the plunger until buffer drips out
4. Push the angled end of the tubing into the device
5. Before placing the bead syringe, turn on the magnetic mixer for stirring disc (speed 25-30). Carefully rotate bead syringe by hand to mix up the beads in the lysis buffer.
6. Place bead syringe in pump and secure. Make sure the magnetic disc is circling up and down vertically in the syringe to keep the beads well mixed.
7. Using the screen, hold the free tube over the wase collection, then slowly move the pusher to the plunger until buffer drips out
8. Push the angled end of the tubing into the device



Source: http://mccarrolllab.org/download/905/

21 Run Pumps for Droplet Formation

1. Start oil pump
2. Start bead pump
3. Start cells/sample pump

22 Check for Droplet Formation

1. Have outflow line go to waste
2. Watch the beads move from the syringe to the device
3. Once the beads get to the bead chamber in the device, use a clean microscope slide to catch a drop of output
4. Check slide under a different microscope to see if droplets are uniform in size and shape
5. Once droplets are uniform begin collecting

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23 Collect

1. Collect uniform droplets in 15 ml Falcon tubes until cells/sample runs out
2. The pump will sound an alarm once the cell/sample runs out; turn off alarm
3. STOP collecting in the 15 ml Falcon tube immediately (place a waste container under the outflow)

24 Stop pumps

1. Stop all pumps in any order
2. Place the 15 ml Falcon tube with droplets **on ice**

25 When running more than one sample **go to step #20**

Repeat Step 20- Step 24 for each sample

26 Clean up (**AFTER** Reverse Transcription is set up to incubate)

1. Turn off magnetic mixer
2. Remove syringes from pumps; remove tubing from microfluidic device
3. Power off pumps (switches located on the back left of the pump)
4. Cell/sample outflow tubing goes in biohazardous solid waste
5. All other lines can go in regular waste
6. Cell/sample syringes go in biohazardous solid waste
7. All other syringes can go in regular waste
8. Cell/sample needles go in biohazardous sharps waste
9. All other needles go in regular sharps waste

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STAMP Collection

27 **S**ingle cell/nuclei **T**ranscriptomes **A**ttached to **M**icro**P**articles (STAMP)

STAMPs are the barcoded beads with mRNA attached; for stability, work **on ice/keep cold** when working with STAMPs/RNA

Add Maxima H- RTase to RT Mix (Step 10)

Prepare waste and collection tube

- Need one 50 mL Falcon tube for waste (can use same waste from preparing syringes)
- Need one new clean 50 mL Falcon tube for collection
- Need one 30 um Uberstrainer
- Autopipette, 5 mL pipette, and attachment for strainer

50 mL centrifuge tubes
by VWR International
Catalog #: 734-1876

30 um Uberstrainer
by pluriSelect
Catalog #: 43-70030-03

Citation: Song Chen, Blue B. Lake, Sarah Urata, Kun Zhang (04/10/2020). Single Nucleus Drop-seq (snDrop-seq). https://dx.doi.org/10.17504/protocols.io.zmvf466

28 Breakage

1. Be careful not to disturb the oil/droplet band (direct buffer down the side of the 15 mL Falcon tube and control output so there is smooth flow)
2. Before droplet breakage, add **600 µl mineral oil** and incubate in water bath at **72 °C** for **00:05:00**
3. Let sit on ice for **00:05:00**
4. Remove the oil from the bottom of the 15 mL Falcon tube
5. Add **5 ml cold 6X SSC**
6. Add **1 ml Perfluorooctanol (PFO)** directly to the oil/droplet band to break the droplets; dispense in a circular motion directly over/onto the droplet bad
7. Gently roll/rotate the 15 mL Falcon tube on ice to help break the droplets

Mineral Oil
by Millipore Sigma
Catalog #: M5904

Perfluorooctanol
by Sigma Aldrich
Catalog #: 370533

29 Filter/Collection

Set up the 30 um Uberstrainer over the waste tube

1. Place strainer over waste tube (goal: save beads, discard supernatant)
2. Attach autopipette attachment to strainer
3. Angle/tilt tube so that no liquid will go up through the autopipette when using it for a vacuum
4. Use **1 ml cold 6X SSC** to moisten the filter

Filter the beads (over waste tube)

1. Pass the aqueous (top layer) phase through the strainer
2. Pass the organic (bottom layer) phase through the strainer
3. Use a couple mililiters cold 6X SSC to rinse the 15 mL Falcon tube to try and get all the beads from the sides; pass the rinse through the strainer
4. Rinse strainer/filter twice with cold 6X SSC

Collect the beads (over new/clean 50 mL Falcon tube)

1. Carefully reverse the strainer over a new/clean 50 mL Falcon tube
2. Wash strainer with **1 ml cold 6X SSC** . Repeat four more times (5 washes total)
3. Visually inspect the strainer to make sure all the beads have come off it

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30 Spin Down

1. Using a **4 °C centrifuge** , spin down the 50 mL Falcon tube at 1000xg for **00:01:00**
2. Carefully remove, supernatant, leaving approximately **1 ml cold 6X SSC**

31 Transfer to 1.5 mL tube

1. Resuspend beads in remaining **1 ml cold 6X SSC**
2. Transfer to a new/clean 1.5 mL microfuge tube
3. Spin down (1000xg for **00:01:00**)
4. Wash twice with **1 ml cold 6X SSC**
5. Wash with **300 µl 5X RT buffer**
6. Remove as much of the 5X RT buffer as possible without taking up any beads

Maxima 5X RT Buffer
by Thermo Fisher Scientific
View

Reverse Transcription

32 This step generates cDNA strands on the RNA hybridized to the bead primers. One RT mix is sufficient for processing approximately 90,000 beads.

1. Add **200 µl RT Mix** to the beads
2. Incubate at **Room temperature with rotation** for **00:30:00**
3. Incubate at **42 °C with rotation** for **01:30:00**
4. Wash beads once with **1 ml TE-SDS**
5. Wash beads twice with **1 ml TE-TW**
6. If proceeding to Exonuclease I treatment, wash once with **1 ml 10 mM Tris pH 8.0**

Stopping point: if stopping, stop at TE-TW wash step. Beads can be stored at **4 °C in TE-TW**

Citation: Song Chen, Blue B. Lake, Sarah Urata, Kun Zhang (04/10/2020). Single Nucleus Drop-seq (snDrop-seq). https://dx.doi.org/10.17504/protocols.io.zmvf466

Exonuclease I Treatment

33 This step chews back the excess bead primers that did not capture an RNA molecule. One Exonuclease Mix is sufficient for processing approximately 90,000 beads.

1. After washing once with **1 ml 10 mM Tris pH 8.0** , resuspend in **200 µl Exonuclease Mix**
2. Incubate at **37 °C with rotation** for **00:45:00**
3. Wash beads once with **1 ml TE-SDS**
4. Wash beads twice with **1 ml TE-TW**
5. If proceeding to PCR, wash once with **1 ml H2O**

Stopping point: if stopping, stop at TE-TW wash step. Beads can be stored at **4 °C in TE-TW**

PCR

34 This steps utilizes PCR to amplify the cDNA constructed during the Reverse Transcription step.

Bead Count

1. After washing once with **1 ml H2O** , spin down to pellet beads
2. Remove supernatant
3. Add **1 ml H2O**
4. Mix well by pipette to evenly resuspend the beads
5. Quickly remove **20 µl** and pipette into a Fuchs-Rosenthal hemocytometer chamber
6. Count all 16 boxes

35 Calculate number of beads to split into PCR tubes

1. Calcuate the concentraion (beads/ul) = (#beads counts/16) x 5
2. Apportion 2,000 beads into each PCR tube (want approximately 100 STAMPs per PCR tube)

Citation: Song Chen, Blue B. Lake, Sarah Urata, Kun Zhang (04/10/2020). Single Nucleus Drop-seq (snDrop-seq). https://dx.doi.org/10.17504/protocols.io.zmvf466

36 Prepare Master Mix

Master Mix (**50 µl per PCR reaction**)

24.6 µl H2O

Nuclease-free H2O
by Life Technologies
Catalog #: AM9930

0.4 µl 100 uM SMART PCR Primer

SMART PCR primer: AAGCAGTGGTATCAACGCAGAGT
by Integrated DNA Technologies
View

25 µl 2x Kapa HiFi Hotstart ReadyMix

Kapa HiFi Hotstart Readymix
by Kapa Biosystems
Catalog #: KK2601

Citation: Song Chen, Blue B. Lake, Sarah Urata, Kun Zhang (04/10/2020). Single Nucleus Drop-seq (snDrop-seq). https://dx.doi.org/10.17504/protocols.io.zmvf466

37 PCR

1. Spin down tubes
2. Add **50 µl PCR Master Mix** to each reaction
3. Mix well by pipette
4. Run the following program on a thermocycler

95 °C for **00:03:00**

4 cycles of

98 °C for **00:00:20**

65 °C for **00:00:45**

72 °C for **00:03:00**

13 cycles of

98 °C for **00:00:20**

67 °C for **00:00:20**

72 °C for **00:03:00**

Then

72 °C for **00:05:00**

4 °C forever

Purification and Quantification

38 Magnetic Bead Purification

1. Using either KAPA PureBeads or AMPure XP beads, add **30 µl room temperature magnetic beads** to each PCR sample (this is a 0.6x beads to samples ratio based on volume)
2. Purify according to manufacturer's instructions
3. Elute in **10 µl H2O**

Agencourt AMPure XP beads
by Beckman Coulter
Catalog #: A63881

Citation: Song Chen, Blue B. Lake, Sarah Urata, Kun Zhang (04/10/2020). Single Nucleus Drop-seq (snDrop-seq). https://dx.doi.org/10.17504/protocols.io.zmvf466

39 Quantification

Can be performed by preferred method (qPCR, Qubit Assay, BioAnalyzer, Tapestation); we used Qubit Assay.

1. Use **2 µl sample input** for this quantification
2. Quantify according to manufacturer's instructions

Qubit 4 Fluorometer
by Thermo Fisher Scientific
Catalog #: Q33238

Qubit dsDNA HS Assay Kit
by Thermo Fisher Scientific
Catalog #: Q32854

The yield for 2000 beads generated from a 50 cell/ul total yield should be approximately 1-100ng.

QC Cutoff: > 1ng total

40 Amplified cDNA Gel

1. Confirm average size of cDNA library via gel electrophoresis
2. Average size should be between 1300-2000 bp

Tagmentation

41 This step simultaneously tags and fragments the cDNA library using transposomes

Citation: Song Chen, Blue B. Lake, Sarah Urata, Kun Zhang (04/10/2020). Single Nucleus Drop-seq (snDrop-seq). https://dx.doi.org/10.17504/protocols.io.zmvf466

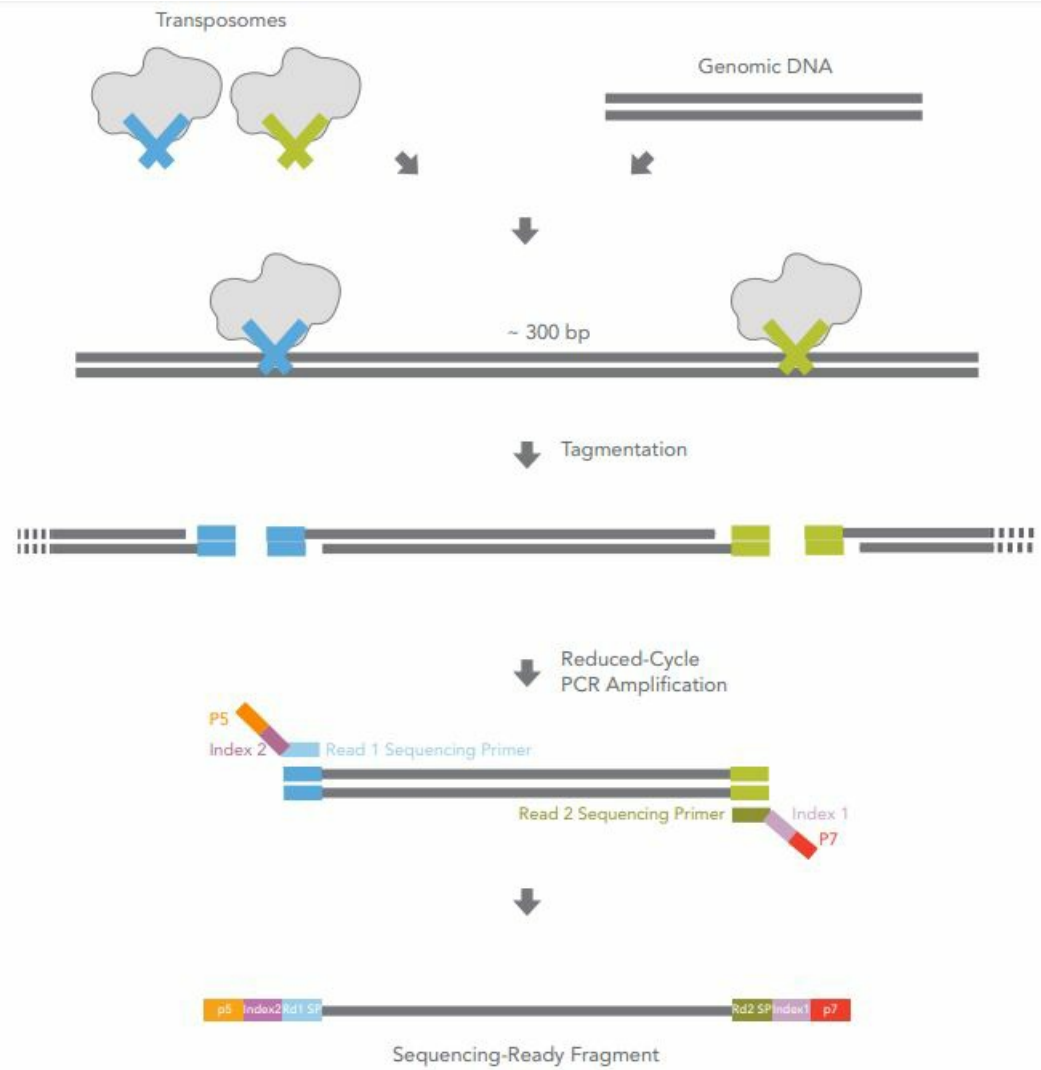

Figure 2: Nextera Library Preparation Biochemistry—Nextera chemistry simultaneously fragments and tags DNA in a single step. A simple PCR amplification then appends sequencing adapters and sample indexes to each fragment.

Source: https://www.illumina.com/content/dam/illumina-marketing/documents/products/datasheets/datasheet_nextera_dna_sample_prep.pdf

Citation: Song Chen, Blue B. Lake, Sarah Urata, Kun Zhang (04/10/2020). Single Nucleus Drop-seq (snDrop-seq). https://dx.doi.org/10.17504/protocols.io.zmvf466

42 Nextera Kit

1. Preheat a thermocycler to **55 °C**
2. Working in a cold rack/on ice: for each sample, combine **600 pg purified cDNA** with **H2O** in a total volume of **5 µl**
3. To each tube, add **10 µl Nextera TD buffer "Tagment DNA"** and

   5 µl Amplicon Tagment enzyme "Amplicon Mix" (the total volume of the reaction is now **20 µl**)
4. Mix by pipette approximately 5 times
5. Spin down
6. Incubate at **55 °C** for **00:05:00**
7. Add **5 µl Neutralization Buffer** (the total volume of the reaction is now **25 µl**)
8. Mix by pipette approximately 5 times
9. Spin down (bubbles are normal)
10. Incubate at **Room temperature** for **00:05:00**

Nextera XT DNA Library Preparation Kit
by Illumina, Inc.
Catalog #: FC-131-1096

Citation: Song Chen, Blue B. Lake, Sarah Urata, Kun Zhang (04/10/2020). Single Nucleus Drop-seq (snDrop-seq). https://dx.doi.org/10.17504/protocols.io.zmvf466

43 PCR work up of tagmented library

Prepare PCR Master Mix (**24 µl per PCR reaction**)

15 µl Nextera PCR mix

Nextera XT DNA Library Preparation Kit
by Illumina, Inc.
Catalog #: FC-131-1096

8 µl H2O

Nuclease-free H2O
by Life Technologies
Catalog #: AM9930

1 µl 10 uM New-P5-SMART PCR hybrid oligo

New-P5-SMART PCR hybrid oligo: AATGATACGGCGACCACCGAGATCTA CACGCCT GTCCGCGGAAGCAGTGGTATCAACGC AGAGT* A*C
by Integrated DNA Technologies
View

44 PCR

1. Add **24 µl Tagmentation PCR Master Mix** to each tube
2. To each individual tube, add **1 µl 10 uM Nextera N7XX oligo** The total volume of the reaction is now **50 µl**
3. Mix well and run the following program on a thermocycler

95 °C for **00:00:30**

12 cycles of

95 °C for **00:00:10**

55 °C for **00:00:30**

72 °C for **00:00:30**

Then

72 °C for **00:05:00**

4 °C forever

Citation: Song Chen, Blue B. Lake, Sarah Urata, Kun Zhang (04/10/2020). Single Nucleus Drop-seq (snDrop-seq). https://dx.doi.org/10.17504/protocols.io.zmvf466

Purification and Quantification

45 Magnetic Bead Purification

1. Using either KAPA PureBeads or AMPure XP beads, add **30 µl room temperature magnetic beads** to each PCR sample (this is a 0.6x beads to samples ratio based on volume)
2. Purify according to manufacturer's instructions
3. Elute in **12 µl** to **15 µl H2O**

Agencourt AMPure XP beads
by Beckman Coulter
Catalog #: A63881

46 2nd Magnetic Bead Purification

1. Using either KAPA PureBeads or AMPure XP beads, perform a 0.6x purification
2. Purify according to manufacturer's instructions
3. Elute in **12 µl** to **15 µl H2O**

Agencourt AMPure XP beads
by Beckman Coulter
Catalog #: A63881

Citation: Song Chen, Blue B. Lake, Sarah Urata, Kun Zhang (04/10/2020). Single Nucleus Drop-seq (snDrop-seq). https://dx.doi.org/10.17504/protocols.io.zmvf466

47 Quantification

Can be performed by preferred method (qPCR, Qubit Assay, BioAnalyzer, Tapestation); we used Qubit Assay.

1. Use **1 µl sample input** for this quantification
2. Quantify according to manufacturer's instructions
3. Convert ng/ul to nM

Qubit 4 Fluorometer
by Thermo Fisher Scientific
Catalog #: Q33238

Qubit dsDNA HS Assay Kit
by Thermo Fisher Scientific
Catalog #: Q32854

The yield for 2000 beads generated from a 50 cell/ul final cell concentration should be approximately 400-1000 pg/ul.

QC Cutoff >1ng

48 Sequencing Library Gel

1. Confirm average size of Sequencing library via gel electrophoresis
2. Average size should be between 500-680 bp

Smaller-sized libraries will have more polyA reads; larger libraries may have lower sequence cluster density and cluster quality. Although the target size is 500-680 bp, the range can be as broad as 420-700 bp.

Citation: Song Chen, Blue B. Lake, Sarah Urata, Kun Zhang (04/10/2020). Single Nucleus Drop-seq (snDrop-seq). https://dx.doi.org/10.17504/protocols.io.zmvf466

Sequencing

49 MiSeq Sequencing - **QC for estimation of library quality and number of nuclei captured**

1. Pool, denature, and dilute to loading concentration according to manufacturer's instructions
2. Sequencing specifications
- Read 1: 30 bp
- Read 2: 75~100 bp
- Read 1 Index: 8 bp
- Custom Read 1 primer

Hiseq 2500 Sequencing

1. Combine 8-12 snDrop-seq libraries to make a 10 µl library pool at 3 nM for denaturation.
2. After final dilution, load a combined library at 12 pM to the sequencer
- Read 1: 30 bp
- Read 2: 75~100 bp
- Read 1 Index: 8 bp
- Custom Read 1 primer

Custom Read 1 primer:
GCCTGTCCGCGGAAGCAGTGGTATCA
ACGCAG AGTAC
by Integrated DNA Technologies
View

MiSeq v3 (150 cycle) Kit
by Illumina, Inc.
Catalog #: MS-102-3001

50 snDrop-seq data processing

Mapping, demultiplexing and QC processing: https://github.com/chensong611/Dropseq_pipeline
- Paired-end reads are removed if read 1 had more than four non-T bases in the last ten bases (to remove all non-poly(T)-captured contaminated reads)
- Paired-end reads are removed if read 1 had one or more bases with a poor quality score (<10)
- The right mate of each read pair is trimmed to remove any portion of the SMART adaptor sequence or any large stretches of poly(A) tails (6 consecutive bp or larger)
- The trimmed reads are aligned to the human genome (GENCODE GRCH38) with STAR (e.g. v2.5) with default parameter settings
- Reads that mapped to intronic or exonic regions as per the GENCODE gene annotation are included in gene counts
- Barcode synthesis errors are corrected by inserting N at the last base of the cell barcode for reads in which the first 11 bases of the cell barcode are identical and the last T base of the UMI is the same
- UMI counts for each gene of each nucleus are assigned by collapsing UMI reads that had only 1 edit distance to create a digital expression matrix (genes as rows, cells or nuclei as columns)

Citation: Song Chen, Blue B. Lake, Sarah Urata, Kun Zhang (04/10/2020). Single Nucleus Drop-seq (snDrop-seq). https://dx.doi.org/10.17504/protocols.io.zmvf466