DroNc-seq step-by-step

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Abstract

Currently, most single cell protocols require the preparation of a single cell suspension from fresh tissue, a major roadblock to clinical deployment, to archived materials and to certain tissues such as adult brain. In the adult brain the harsh enzymatic dissociation harms the integrity of the cells and their RNA, and biases toward easily dissociated cell types, and is restricted to young animals.

We developed DroNc-seq, a droplet microfluidic and DNA barcoding technique for analysis of RNA profiles of single nuclei from fresh, frozen or lightly fixed tissues at high throughput and low cost. The utility of DroNc-Seq lies in working with hard-to-dissociate, frozen and/or archived tissues. To demonstrate the utility of this technique, we sequenced over 39 thousand nuclei from mouse and human archived brain samples, including post-mortem human brain tissue from GTEx project.

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Guidelines

[Please note that this protocol is originally published at Protocol Exchange:

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Equipment

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a. Microfluidic chip (see CAD file). The unit in the CAD provided is 1 unit = 1 µm; channel depth on device is 75 µm.
b. Drop-seq microfluidic setup (see reference):
- optical microscope (Olympus IX83)
- Fast camera (Photron SA5)
- Three syringe pumps (KD Scientific, KDS910)
- Magnetic Stirrer (VP Scientific, #710D2)
c. Invitrogen Qubit 3.0 Fluorometer
d. Agilent 2100 Bioanalyzer
e. Illumina NextSeq 500

The protocol workflow is as follows:

1. Beads preparation
2. Cell culture
3. Tissue preservation
4. Nuclei isolation
5. Microfluidics
6. Droplet breakage, washes and reverse transcription (RT)
7. Post RT wash, exonuclease I treatment and PCR
8. WTA library QC and Nextera library prep
9. Sequencing

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References:

Macosko, E. Z. et al. Highly Parallel Genome-wide Expression Profiling of Individual Cells Using

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Nanoliter Droplets. Cell 161, 1202-1214, doi:10.1016/j.cell.2015.05.002 (2015).

Before start

Drop-seq Lysis Buffer (DLB6; a 10 ml stock consists of 4 ml of nuclease-free H2O, 3 ml 20% Ficoll PM-400 (Sigma, Cat # F5415-50ML), 100 µl 20% Sarkosyl (Teknova, Inc., Cat # S3377), 400 µl 0.5M EDTA (Life Technologies), 2 ml 1M Tris pH 7.5 (Sigma), and 500 µl 1M DTT (Teknova, Inc., Cat # D9750), where the DTT is added fresh before every experiment)

Materials

Nuclei EZ lysis buffer EZ PREP NUC-101 by Sigma

RNAlater AM7020 by Thermo Fisher Scientific

PBS buffer 10010023 by Thermo Fisher Scientific

DNAse/RNAse free distilled water 10977023 by Thermo Fisher Scientific

BSA, molecular biology grade, 20 mg/ml B9000S by New England Biolabs

Ficoll PM-400 F5415-50ML by Sigma

Sarkosyl S3377 by Teknova

0.5 M EDTA by Life Technologies

1M Tris pH 7.5 by Sigma

1M DTT D9750 by Teknova

20% PEG solution P4137 by Teknova

10% SDS solution S0287 by Teknova

10% Tween 20 solution T0710 by Teknova

Carrier oil 186-4006 by BioRad Sciences

DAPI D1306 by Thermo Fisher Scientific

6x SSC S0282 by Teknova

1H,1H,2H,2H-Perfluorooctan- 1-ol 647-42- 7 by SynQuest Laboratories

1x Maxima H- RT buffer EP0753 by Fisher Scientific

dNTP 639125 by Takara

RNase Inhibitor 30281-2 by Lucigen

Maxima H-RT enzyme EP0753 by Fisher Scientific

Exonuclease I kit M0293L by New England Biolabs

2x Kapa HiFi Hotstart Readymix KK2602 by Kapa Biosystems

Nextera XT sample prep kit, 96 samples FC-131- 1096 by illumina

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

emplate Switch Oligo, AAGCAGTGGTATCAACGCAGAGTGAATrGrGrG (IDT, custom RNA oligo, HPLC

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purified) by Contributed by users

✓ SMART PCR primer, AAGCAGTGGTATCAACGCAGAGT (IDT, custom DNA oligo, standard desalting) by Contributed by users

✓ P5-PCR hybrid oligo AATGATACGGCGACCACCGAGATCTACACGCCTGTC CGCGGAAGCAGTGGTATCAACGCAGAGT*A*C, (IDT, custom DNA oligo) by Contributed by users

✓ Custom Read1 primer, GCCTGTCCGCGGAAGCAGTGGTATCAACGCAGAGTAC (IDT, custom DNA oligo, standard desalting) by Contributed by users

Cell strainer, 35 µm 352235 by Corning

Cell strainer, 40 µm 43-50040- 03 by pluriSelect

Cell strainer, 100 µm 08-771- 19 by Vwr

Dounce homogenizers D8938-1SET by Sigma

Fuchs-Rosenthal (FR) hemocytometer 22-600- 102 by Vwr

Neubauer Improved (NI) Hemocytometer 22-600- 100 by Life Technologies

3ml syringe BD309657 by BD Biosciences

10 ml syringe (BD309695 by BD Biosciences

26G1/2 sterile needles BD305111 by BD Biosciences

PE tubing BB31695-PE/2 by Scientific Commodities

Flea magnet 782N-6- 150 by VP Scientific

1.5 ml micro-centrifuge tube AM12450 by Ambion

Ampure XP beads A63881 by Beckman Coulter

Qubit dsDNA HS Assay kit Q32854 by Thermo Fisher Scientific

BioAnalyzer High Sensitivity Chip 5067-4626 by Agilent Technologies

✓ Illumina NextSeq 75 by Contributed by users

Protocol

Beads preparation

Step 1.

Wash and filter barcoded beads (Chemgenes, Cat # Macosko-2011-10) as previously described.

Beads preparation

Step 2.

Isolate beads smaller than 40 µm, using a 40 µm cell strainer (PluriSelect, Cat # 43-50040-03).

Beads preparation

Step 3.

Suspend barcoded beads in Drop-seq Lysis Buffer.

NOTES

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Anita Bröllochs 22 Dec 2017

Drop-seq Lysis Buffer (DLB6; a 10 ml stock consists of 4 ml of nuclease-free H2O, 3 ml 20% Ficoll PM-400 (Sigma, Cat # F5415-50ML), 100 µl 20% Sarkosyl (Teknova, Inc., Cat # S3377), 400 µl 0.5M EDTA (Life Technologies), 2 ml 1M Tris pH 7.5 (Sigma), and 500 µl 1M DTT (Teknova, Inc., Cat # D9750), where the DTT is added fresh before every experiment)

Beads preparation

Step 4.

Count beads at 1:1 dilution in 20% PEG solution, using a disposable Fuchs-Rosenthal hemocytometer (VWR, Cat # 22-600-102) and resuspend beads at concentrations ranging between 325,000 and 350,000 per ml.

Cell culture

Step 5.

Cell lines are cultured according to ATCC's instructions. For DroNc-seq, wash cells once with 1x PBS, scrape them with 2 ml nuclease- and protease-free Nuclei EZ lysis or EZ PREP buffer (Sigma, Cat # EZ PREP NUC-101) and process as tissues, described below.

AMOUNT

2 ml Additional info: Nuclease- and protease-free Nuclei EZ lysis or EZ PREP buffer

Tissue preservation

Step 6.

Tissue samples may be flash-frozen on dry ice and stored at -80°C until they are processed for nuclei isolation. To preserve tissue in RNAlater, samples are placed in ice-cold RNAlater (ThermoFisher Scientific, Cat # AM7020) and stored at 4°C overnight.

TEMPERATURE

4 °C Additional info: Storage overnight

Tissue preservation

Step 7.

Remove RNAlater the following day and store samples at -80°C until processing.

TEMPERATURE

-80 °C Additional info: Storage

Nuclei isolation

Step 8.

Use either fresh, frozen or RNAlater fixed tissue or fresh cells as input material.

Nuclei isolation

Step 9.

Prepare Nuclei Suspension Buffer (NSB; consisting of 1x PBS, 0.01% BSA (New England Biolabs, Cat # B9000S) and 0.1% RNAse inhibitor (Clontech, Cat #2313A)).

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Nuclei isolation

Step 10.

Dounce homogenize tissue samples (smaller than 0.5 cm) or cell pellets in 2 ml of ice-cold Nuclei EZ lysis buffer (Sigma, #EZ PREP NUC-101). For brain tissue: grind 20-25 times with pestle A, followed by 20-25 times with pestle B (This may need to be modified for other tissues). Move sample to a 15 ml conical tube, add 2 ml of ice-cold Nuclei EZ lysis buffer and incubate on ice for 5 minutes.

AMOUNT
2 ml Additional info: Ice-cold Nuclei EZ lysis buffer
AMOUNT
2 ml Additional info: Ice-cold Nuclei EZ lysis buffer

ANNOTATIONS
Alexander Chamessian 28 May 2018

It looks like you don't put any RNase inhibitor in the EZ Nuc buffer. You use it as is? Is that correct? Do you feel that, since you used RNAlater, you don't need to have RNase inhibitors during the lysis? What about if using fresh or frozen tissue?

Nuclei isolation

Step 11.

Collect nuclei by centrifugation at 500 x g for 5 minutes at 4°C.

TEMPERATURE
4 °C Additional info: Centrifugation

Nuclei isolation

Step 12.

Discard supernatant and carefully resuspend nuclei in 4 ml of ice-cold Nuclei EZ lysis buffer.

AMOUNT
4 ml Additional info: Ice-cold Nuclei EZ lysis buffer

Nuclei isolation

Step 13.

Incubate on ice for 5 minutes.

Nuclei isolation

Step 14.

Collect nuclei by centrifugation at 500 x g for 5 minutes at 4°C.

TEMPERATURE
4 °C Additional info: Centrifugation

Nuclei isolation

Step 15.

Resuspend isolated nuclei in 4 ml of NSB and collect nuclei by centrifugation at 500 x g for 5 minutes

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at 4°C.

AMOUNT
4 ml Additional info: NSB
TEMPERATURE
4 °C Additional info: Centrifugation

Nuclei isolation

Step 16.

Resuspend isolated nuclei in 1 ml of NSB, and filter through a 35 µm cell strainer (Corning, Cat # 352235). Stain 10 µl of the single nuclei suspension with DAPI (Fisher, Cat # D1306), load on an NI hemocytometer, and count under a microscope. A final concentration of 300,000 nuclei/ml is used for DroNc-seq experiments. Proceed immediately to microfluidic droplet co-encapsulation.

AMOUNT
1 ml Additional info: NSB
AMOUNT
10 µl Additional info: Single nuclei suspension

Microfluidics

Step 17.

Load the nuclei and barcoded bead suspension into 3 ml syringes (BD Scientific, Cat # BD309695) and connect to DroNc-seq microfluidic chip via 26G1/2 sterile needles (BD Scientific, Cat # BD305111) and PE2 tubing (Scientific Commodities, Inc. Cat # BB31695-PE/2).

AMOUNT
3 ml Additional info: Syringes

NOTES
Anita Bröllochs 29 Dec 2017

Note that the bead syringe is loaded onto the syringe pump in an upside down position, along with a flea magnet inside the syringe and constant stirring, using external magnetic stirrer.

Microfluidics

Step 18.

Flow both bead and nuclei suspensions at 1.5 ml/hr each, along with carrier oil (BioRad Sciences, Cat # 186-4006) loaded in 10 ml syringes (BD Scientific, Cat # BD309695) and flown at 16 ml/hr to co-encapsulate single nuclei and beads in 75 µm drops at 4,500 drops/sec and double Poisson loading concentrations.

AMOUNT
10 ml Additional info: Syringes

Microfluidics

Step 19.

Collect resulting emulsion via PE2 tubing into a 50 ml Falcon tube for a period of 22 min each, and incubate at room temperature for up to 45 min before proceeding to break droplets.

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Droplet breakage, washes and reverse transcription (RT)

Step 20.

Emulsion collected after microfluidic co-encapsulation has the droplets cream to the top with clear oil collected under the droplets. Carefully remove the excess clear oil, add 30 ml of 6x SSC (Teknova, Inc., Cat # S0282) into each 50 ml Falcon collection tube, agitate it vigorously, and add 1 ml of 1H,1H,2H,2H-Perfluorooctan-1-ol (SynQuest Laboratories, Cat # 647-42-7).

AMOUNT
30 ml Additional info: 6x SSC
AMOUNT
1 ml Additional info: 1H,1H,2H,2H-Perfluorooctan-1-ol

NOTES
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It is recommended that all washes following this step be performed and the beads temporarily stored on ice.

Droplet breakage, washes and reverse transcription (RT)

Step 21.

Vigorously shake the tubes by hand and centrifuge at 1,000 x g for 1 min.

Droplet breakage, washes and reverse transcription (RT)

Step 22.

Carefully remove the supernatant from each tube and squirt an additional 30 ml of 6x SSC to kick up the beads from the oil-water interface into the aqueous phase.

AMOUNT
30 ml Additional info: 6x SSC

Droplet breakage, washes and reverse transcription (RT)

Step 23.

Remove the beads that were kicked up momentarily into the SSC with a 25 ml pipette and transfer them into a clean 50 ml Falcon tube, leaving the heavier oil behind.

Droplet breakage, washes and reverse transcription (RT)

Step 24.

Centrifuge the newly transferred beads and SSC mix again at 1,000 x g for 1 min; carefully remove the supernatant leaving 1 ml of SSC and bead sediment behind.

Droplet breakage, washes and reverse transcription (RT)

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Step 25.

Carefully transfer remaining SSC and bead mix into a 1.5 ml micro-centrifuge tube (Ambion, Cat # AM12450) and spin it down on a desktop micro-centrifuge for 10 sec to generate a noticeable bead pellet.

Droplet breakage, washes and reverse transcription (RT)

Step 26.

Remove any residual oil that got transferred into the 1.5 ml tube with a p200 pipette with low-retention pipette tip.

Droplet breakage, washes and reverse transcription (RT)

Step 27.

Wash the beads again in 1.5 ml of 6x SSC.

AMOUNT
1.5 ml Additional info: 6x SSC

Droplet breakage, washes and reverse transcription (RT)

Step 28.

Wash the beads again in 300 µl of 5x Maxima H- RT buffer (Fisher, Cat # EP0753). A pellet of barcoded beads in each micro-centrifuge tube should have 130,000 beads.

AMOUNT
300 µl Additional info: 5x Maxima H- RT buffer

Droplet breakage, washes and reverse transcription (RT)

Step 29.

Make a fresh batch of 200 µl RT mix for each barcoded bead aliquot, consisting of: 80 µl H2O, 40 µl Maxima 5x RT Buffer, 40 µl 20% Ficoll PM-400 (Sigma, Cat # F5415-50ML), 20 µl 10 mM dNTP (Takara Bio, Cat # 639125), 5 µl RNase Inhibitor (Lucigen, Cat # 30281-2), 10 µl Maxima H-RT enzyme (Fisher, Cat # EP0753), and 5 µl 100 µM Template Switch Oligo, AAGCAGTGGTATCAACGCAGAGTGAATrGrGrG (IDT, custom RNA oligo, HPLC purification).

AMOUNT
80 µl Additional info: H2O
AMOUNT
40 µl Additional info: Maxima 5x RT Buffer
AMOUNT
40 µl Additional info: 20% Ficoll PM-400 (Sigma, Cat # F5415-50ML)
AMOUNT
20 µl Additional info: 10 mM dNTP (Takara Bio, Cat # 639125)
AMOUNT
5 µl Additional info: RNase Inhibitor (Lucigen, Cat # 30281-2)
AMOUNT

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10 µl Additional info: Maxima H-RT enzyme (Fisher, Cat # EP0753)

AMOUNT

5 µl Additional info: 100 µM Template Switch Oligo, AAGCAGTGGTATCAACGCAGAGTGAATrGrGrG (IDT, custom RNA oligo, HPLC purification)

Droplet breakage, washes and reverse transcription (RT)

Step 30.

After the supernatant is carefully removed from each bead pellet, add 200 µl of the above RT mix into each tube, and incubate it under gentle rocking or tumbling for 30 min at room temperature, and then at 42°C for 1.5 hr in a rotisserie-style hybridization oven, for a total of two hours.

AMOUNT

200 µl Additional info: RT mix

Post RT wash, exonuclease I treatment and PCR

Step 31.

Post RT, each bead has cDNA barcoded with the bead's unique barcode (BC) bound onto it, also referred to as a STAMP6. Wash each STAMP pellet with 1 ml of TE buffer containing 0.5% SDS (TE-SDS).

AMOUNT

1 ml Additional info: TE buffer containing 0.5% SDS (TE-SDS)

Post RT wash, exonuclease I treatment and PCR

Step 32.

Wash each STAMP pellet with 1 ml of TE buffer containing 0.01% Tween-20 (TE-TW). (1/2)

AMOUNT

1 ml Additional info: TE buffer containing 0.01% Tween-20 (TE-TW)

Post RT wash, exonuclease I treatment and PCR

Step 33.

Wash each STAMP pellet with 1 ml of TE buffer containing 0.01% Tween-20 (TE-TW). (2/2)

AMOUNT

1 ml Additional info: TE buffer containing 0.01% Tween-20 (TE-TW)

Post RT wash, exonuclease I treatment and PCR

Step 34.

Wash each STAMP pellet with 1 ml of 10 mM Tris pH 8.0.

AMOUNT

1 ml Additional info: 10 mM Tris pH 8.0

Post RT wash, exonuclease I treatment and PCR

Step 35.

Spin down to remove all supernatant and treat the STAMPs with exonuclease I (New England Biolabs, Cat # M0293L) as follows: add 20 µl of Exo I buffer.

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AMOUNT

20 µl Additional info: Exo I buffer

Post RT wash, exonuclease I treatment and PCR

Step 36.

Add 170 µl of RNAse free water.

AMOUNT

170 µl Additional info: RNAse free water

Post RT wash, exonuclease I treatment and PCR

Step 37.

Add 10 µl of Exo I enzyme.

AMOUNT

10 µl Additional info: Exo I enzyme

Post RT wash, exonuclease I treatment and PCR

Step 38.

Mix well by pipetting up and down, and incubate for 45 min at 37°C under rotation to remove all unextended primers.

TEMPERATURE

37 °C Additional info:

Post RT wash, exonuclease I treatment and PCR

Step 39.

Wash the pellet with 1 ml of TE buffer containing 0.5% SDS (TE-SDS).

AMOUNT

1 ml Additional info: TE buffer containing 0.5% SDS (TE-SDS)

NOTES

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The pellet is washed with TE-SDS and TE-TW washes (as described in Steps 31-34), followed by a round of wash the pellet in 1 ml of RNAse free water. You may pool beads from multiple collections of a given sample at this point.

Post RT wash, exonuclease I treatment and PCR

Step 40.

Wash the pellet with 1 ml of TE buffer containing 0.01% Tween-20 (TE-TW). (1/2)

AMOUNT

1 ml Additional info: TE buffer containing 0.01% Tween-20 (TE-TW)

Post RT wash, exonuclease I treatment and PCR

Step 41.

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Wash the pellet with 1 ml of TE buffer containing 0.01% Tween-20 (TE-TW). (2/2)

AMOUNT
1 ml Additional info: TE buffer containing 0.01% Tween-20 (TE-TW)

Post RT wash, exonuclease I treatment and PCR

Step 42.

Wash the pellet with 1 ml of 10 mM Tris pH 8.0.

AMOUNT
1 ml Additional info: 10 mM Tris pH 8.0

Post RT wash, exonuclease I treatment and PCR

Step 43.

Wash the pellet in 1 ml of RNAse free water.

AMOUNT
1 ml Additional info: RNAse free water

NOTES
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You may pool beads from multiple collections of a given sample at this point.

Post RT wash, exonuclease I treatment and PCR

Step 44.

Resuspend pellet in 1 mL of H2O, and count them, by mixing 10 µl of bead suspension with an equal volume of 20% PEG solution.

AMOUNT
1 ml Additional info: H2O
AMOUNT
10 µl Additional info: Bead suspension
AMOUNT
10 µl Additional info: 20% PEG solution

Post RT wash, exonuclease I treatment and PCR

Step 45.

Resuspend aliquots of 5,000 beads in a PCR mix each consisting of 24.6 µl H2O, 0.4 µl 100 µM SMART PCRprimer, AAGCAGTGGTATCAACGCAGAGT (IDT, custom DNA oligo, standard desalting purification), and 25 µl 2x Kapa HiFi Hotstart Readymix (Kapa Biosystems, Cat # KK2602).

AMOUNT
24.6 µl Additional info: H2O
AMOUNT
0.4 µl Additional info: 100 µM SMART PCRprimer, AAGCAGTGGTATCAACGCAGAGT (IDT, custom DNA oligo, standard desalting purification)

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AMOUNT

25 µl Additional info: 2x Kapa HiFi Hotstart Readymix (Kapa Biosystems, Cat # KK2602)

Post RT wash, exonuclease I treatment and PCR

Step 46.

Amplify the samples in separate wells on a skirted PCR plate, using the Eppendorf Thermocycler (Part # EP-950030020).

i. Amplify Mouse PCR samples using the following PCR steps: 95°C for 3 min; then 4 cycles of: 98°C for 20 sec, 65°C for 45 sec, 72°C for 3 min; then 10 cycles of: 98°C for 20 sec, 67°C for 20 sec, 72°C for 3 min; and finally, 72°C for 5 min. Pool amplified mouse PCR products in batches of 4 wells or 16 wells.

ii. Amplify Human PCR samples with either the previously mentioned PCR steps, or the following PCR steps: 95°C for 3 min; then 4 cycles of: 98°C for 20 sec, 65°C for 45 sec, 72°C for 3 min; then 12 cycles of: 98°C for 20 sec, 67°C for 20 sec, 72°C for 3 min; and finally, 72°C for 5 min. Pool amplified human PCR products in batches of 4 wells (16 total PCR cycles) or 16 wells (14 total PCR cycles).

Post RT wash, exonuclease I treatment and PCR

Step 47.

Combine the 5,000 STAMP aliquots of each well in a 1.5 ml Eppendorf tube and clean with 0.6X SPRI beads (Ampure XP beads, Beckman Coulter, Cat # A63881).

NOTES

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Note that the total number of PCR wells from a single sample depends on the number of STAMPs collected in a DroNc-seq run from a given input of nuclei. A user may access the pool of STAMPs in different ways, depending on the number of nuclei they wish to retrieve and their sequencing setup. In particular, a user would typically access the pool of STAMPs once or more, each time taking only a portion of the STAMPs to generate a library, and repeat the process if more nuclei are desired. For our mouse and human brain samples, it was optimal to pool 20,000 STAMPs in each PCR reaction and then to pool 4 PCR wells together for the library preparation step. Depending on the amount of desired reads per nucleus and the sequencing yield, a user may pool a higher number of PCR wells in a single Nextera library, as we demonstrate here using 16-32 wells.

WTA library QC and Nextera library prep

Step 48.

Quantify purified cDNA using Qubit dsDNA HS Assay kit (ThermoFisher Scientific, Cat # Q32854) and BioAnalyzer High Sensitivity Chip (Agilent, Cat # 5067-4626).

WTA library QC and Nextera library prep

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Step 49.

Use 550 pg of each sample library for fragmentation, tagging and amplification using the Nextera XT sample prep kit, 96 samples (Illumina, Cat # FC-131-1096), and custom primer, AATGATACGGCGACCACCGAGATCTACACGCCTGTCCGCGGAAGCAGTGGTATCAACGCAGAGT*A*C , (IDT, custom DNA oligo, HPLC purification) that enable selective amplification of the 3’ end, according to manufacturer’s instructions.

WTA library QC and Nextera library prep

Step 50.

Quantify Nextera libraries again with Qubit dsDNA HS Assay kit and BioAnalyzer High Sensitivity Chip.

Sequencing

Step 51.

The libraries (at 2.2 pM (mouse, 16 wells pool), 2.7 pM (mouse, 4 wells pool) and 2.3 pM (human)) were sequenced on an Illumina NextSeq 500. We used NextSeq 75 cycle kits to sequence paired-end reads as follows: 20 bp (Read 1), 60 bp (Read 2), and 8 bp for Index 1, with Custom Read1 primer, GCCTGTCCGCGGAAGCAGTGGTATCAACGCAGAGTAC (IDT, custom DNA oligo, standard desalting), according to Illumina loading instructions.

Sequencing

Step 52.

The sequencing cluster density and percent passing filter number from different experiments vary according to the quality of nuclei samples used, but were optimized at around a cluster density of 220 and a 90% passing filter.

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