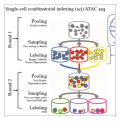

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sciMAP-ATAC

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ABSTRACT

High-throughput single cell genomic assays resolve the heterogeneity of cell states in complex tissues, however, the spatial orientation within the network of interconnected cells is lost. As cell localization is a necessary dimension in understanding complex tissues and disease states, we present a tool for highly scalable spatially-resolved single cell profiling of chromatin state. We use high density multiregional sampling to perform single-cell combinatorial indexing on Microbiopsies Assigned to Positions for the Assay for Transposase Accessible Chromatin (sciMAP-ATAC) to produce single-cell data of equivalent quality to non-spatial single-cell ATAC-seq.

EXTERNAL LINK

doi: https://doi.org/10.1101/407668

MATERIALS

NAME	CATALOG #	VENDOR
Magnesium Chloride	AC223210010	Fisher Scientific
IGEPAL-CA630	I3021 SIGMA-ALDRICH	Sigma Aldrich
Triton X-100	T8787-50ML	Sigma Aldrich
Tween-20	P-7949	Sigma-aldrich
Sodium Chloride	S271-3	Fisher Scientific
Agencourt Ampure XP	A63880	Beckman Coulter
4,6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI)	D1306	Thermo Fisher Scientific
Embedding base molds	22-363-553	Fisher Scientific
Jung tissue freezing medium (Leica Microsystems) or OCT compound (TissueTek)		
Cell strainer, 35 µm	352235	Corning
Pierce Preotease Inhibitor Tablets, EDTA-Free	A32955	Thermo Fisher Scientific
Tris-HCl	AM9855	Life Technologies
Superfrost Plus Microscope Slies	4951PLUS4	Thermo Fisher Scientific
1X PBS, cell culture grade		Thermo Fisher Scientific
Potassium Chloride	P9541	Sigma Aldrich
EDTA	AM9261	Invitrogen - Thermo Fisher
Qiagen Protease	NC9221823	Fisher Scientific
Pitstop 2	SML1169-5MG	Sigma Aldrich
Nextera DNA Flex Library Prep	20018705	Illumina, Inc.

NAME	CATALOG #	VENDOR
QIAquick PCR Purification Kit	28106	Qiagen
Uniquely Indexed Transposomes	View	
Sci- Barcoded PCR Primers	View	
Pitstop 2	SML1169	Sigma Aldrich

MATERIALS TEXT

Tween-20: working stock is 10% (100X). Aliquots are stored at 4C.
IGEPAL-630: Prepare 10% (v/v) stock made with diH20, store at Room Temperature (RT).
DAPI: Resuspend to 5 mg/mL in diH20. Aliquot and store at -20C.
Pitstop2: Resuspend in 3mM in DMSO. Aliquot and store at -20C.

Supplies List:

- 96-well PCR plates (Eppendorf, 951020427)
- 35 um cell strainer (VWR, 21008-948)
- High Sensitivity DNA Chip (Agilent, 5067-4627)

Instrument List:

- Table top centrifuge cooled to 4C with rotors for spinning 1) 96-well plates, and 2) 15 mL falcon tubes at 600 rcf
- Fluorescence Activated Cell Sorter (FACS), we use Sony SH800S
- Thermomixer with 96 well plate adapter (55C incubations at 300 rpm), we use Eppendorf Themomixer C
- Real-Time PCR instrument (Bio-Rad CFX Connect)
- DNA fluorometer or spectrophotometer (Qubit Fluorometer 2.0 is used in this protocol)
- Agilent Bioanalyzer
- Sequencing: NextSeq 500 using custom chemistry protocol

BEFORE STARTING

Cryopreserved tissue sections: Prepare prior to sp-sciATACseq protocol start. Refer to "Cryopreserved tissue sectioning" protocol

Uniquely indexed transposomes (8 uM): Prepare and load prior to sp-sciATACseq protocol start. Refer to "sci Transposase Loading" protocol.

Sp-sci barcoded PCR primers: Prepare prior to sp-sciATACseq protocol start. Refer to "sci Barcoded PCR Primer Preparation" protocol.

Prepare Nuclei Isolation Buffer

1

Construct 50mL **Nuclei Isolation Buffer** (NIB):

Final Concentration	Stock Concentration	Volume of Stock
10 mM Tris HCl, pH 7.5	1M Tris-HCl, pH7.5	500 uL
10 mM NaCl	5M NaCl	100 uL
3mM MgCl2	1M MgCl2	150 uL
0.1 % Igepal	10% Igepal	500 uL
0.1 % Tween	10% Tween	500 uL
ddH20		to 50mL (add 48.25mL)

OPTIONAL: To prevent protease degradation, we also add 2 tablets of Pierce Preotease Inhibitor Tablets, EDTA-Free to NIB following construction. We then vortex to fully dissolve tablets.

NIB is stable at **4 °C** for at least 1 month without noticable degradation in library quality or nuclei dissociation ability.

Store NIB on ice throughout nuclei dissociation and preparation of tagmentation plates.

Isolate nuclei

2 **Nuclei from cryopreserved histological sections**

If sample is sourced from microbiopsy of a cryopreserved histological section, dissociate cells using NIB incubation and trituration (described below).

Note
Isolation of nuclei is dependent on the sample being used. And optimization should be performed. Below we list two example nuclei isolation protocols to act as general use for cell culture and primary tissue samples. Tissue should follow a dounce homogenization protocol, while liquid cell cutures can be pelleted and resuspended directly in NIB.

This protocol is optimized for brain tissue microbiopsies. Additional optimization may need to be performed for other tissues.

1. Prepare 96-well plate(s) for microbiopsy punches
- Pipette 100 uL NIB into each well. Number of wells corresponds to number of punches to be collected.
- Seal plate and store on ice until ready to collect microbiopsies

2. Prepare instruments & tissue for collecting microbiopsies.

- Transfer cryopreserved tissue sections from -80C freezer on to dry ice in an insulated container
- Load Palkovitz punch handle with selected diameter punch (options: 250 um, 500 um, 750 um, 1 mm, 1.25 mm)
- Prechill Palkovitz punch by placing the punch in dry ice

3. Collect microbiopsies **in a cryostat at -20C**
- Place tissue cryosection slide in cryostat and allow ~1 min to acclimate
- Locate region of interest and collect punch
- Deposit punch in well of 96-well plate by depressing punch plunger. (Ensure that punch enters well)
- Repeat for each region to be resected. Place each new punch in new well
- Reseal 96-well plate(s)

Note: Keep a record of 1) slide number, 2) punch location, and 3) well ID for each punch.
Annotating image at cryostat works well.

4. Dissociate and wash microbiopsies
- Shake plate on ice for 1 hour at 80 rpm
- Using a multi-channel pipettor, triturate each well 30x.

Note: Pipette gently in order to reduce bubbles and to prevent nuclei shearing

- Spin down plate for 10 min at 500 rcf at 4C
- Using a multi-channel pipettor, aspirate 90 uL of supernatant.

Note: Pellet will not be visible. Be careful to not touch sides of bottom while drawing off supernatant.

5. Dilute microbiopsy nuclei to desired concentration

Note: We find that for microbiopsy punches from 200 um thick tissue /250 um biopsy punch results in (thousand nuclei):
Min: 6, 1st Q: 12, Median: 15, Mean: 16.85, 3rd Q: 22.25, Max: 29
We want 10 uL nuclei well. Each punch dissociation can be split into 4 wells (4.2K nuclei/rxn).
Therefore, we want 40 uL of 4,200 nuclei/10 uL:
C1V1 = C2V2
(1,685 nuclei/uL)(10uL) = (421.25 nuclei/uL)(**x** uL)
x = 40 uL
Volume to add: 40 uL - 10 uL (residual volume) = **30 uL**

Final concetration of Pitstop 2 should be 70 uM in 40 uL of resuspended nuclei. Therefore:
C1V1 = C2V2
(3000 uM)(**x** uL) = (70 uM)(30 uL); x = 1.43 uL

Therefore, for each well, add: (1.4 uL 3 mM Pitstop 2 + 28.6 uL NIB) = 30 uL of 70 uM Pitstop 2 NIB

This should be done by making a master-mix. Given 1 plate (96 wells), prepare a master-mix for 120 wells:
(168 uL 3mM Pitstop 2 + 3,432 uL NIB) = 3600 uL of 70 uM Pitstop 2 NIB, for one plate.

- Prepare 70 uM Pitstop 2 + NIB master mix: For one plate, combine 168 uL 3 mM Pitstop2 & 3,432 uL NIB
- Add 30 uL of 70 uM Pitstop 2 NIB master mix to each well and triturate to resuspend cells

6. Split punches into multiple plates
- Split 40 uL of resuspend cells into 4 new 96-well (DNA/protein Lo-bind) plates with 10uL diluted cells/well.

Note: Make sure to keep well ID consistent between plates.

96-plex Tagmentation

3 **Prepare tagmentation plate**

Add the following reagents to diluted nuclei in 96-well plate(s) (DNA and Protein Lo-bind):

Prepare 70uM Pitstop 2 + 2X TD buffer mastermix for one plate: 28 uL 3mM Pitstop2 + 1,200 uL 2X TD

Add **10 µl 70uM Pitstop 2/TD buffer (2X)** to each well

Add **1 µl 8uM uniquely indexed transposase** to each well

Spin down plate for **00:01:00 min** at 500 rcf at **4 °C**

4 **Tagmentation**

Seal plate and incubate at **55 °C with gentle shaking (300 rpm on themomixer)** for **00:15:00**

Place plate on ice immediately to stop reaction.
- Keep samples on ice to prevent over-transposistion and nuclei lysis.

5 **Pool all wells for second sort**

Pool all wells into 15mL conical tube, while maintaining everything on ice.

Add 2uL/per mL pooled sample of DAPI (5mg/mL) and bring to sorter for second sort.

96-plex PCR

6 **Preparing Second Plate of Transposase Neutralization Buffer (8.5uL/well):**

Final Concentration	Stock Concentration	Volume of Stock
0.59 mg/mL	20 mg/mL BSA	0.25 uL
0.059% (w/v)	1% SDS (w/v)	0.5 uL
to 8.5 uL	ddH2O	7.75 uL

Per well reagent volumes.

Add 2.5 uL of 10 uM i5 Indexed PCR Primer and 2.5 uL of 10 uM i7 Indexed PCR Primer prior to sort.

7 Flow sort single nuclei based on DAPI gate

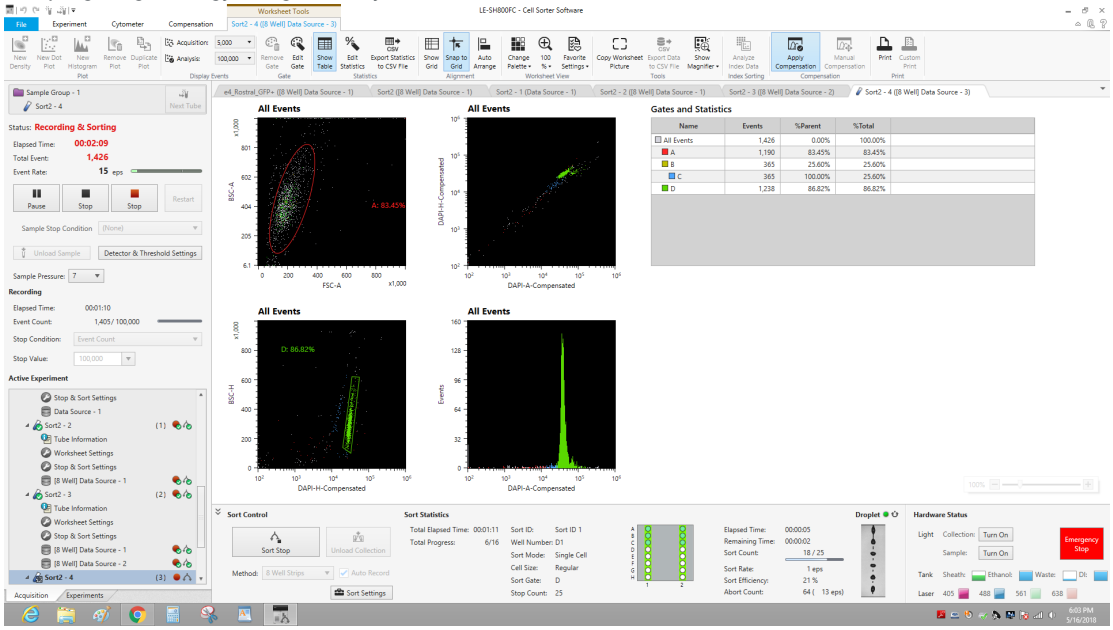
Sort **X** nuclei per well (**X** is dependent on number of wells tagmented in first sort, as a linear trend)
• 96 wells (1 plate) = 22 nuclei/well for PCR
• 144 wells (1 and 1/2 plates) = 33 nuclei/well for PCR
• 192 wells (2 plates) = 44 nuclei/well for PCR etc...

Using the same gates as first sort, sort **X** nuclei per well into prepared second plate with modified sort settings:

- "Single cell" rather than "Normal"
  This leads to a higher abort count (less efficient sorting) but is more precise in quantification

- Keep sorted samples on ice to prevent transposases cross-reacting with other nuclei.

Example gating strategy using the Sony SH800 Flow sorter:



Spin down plate at 500 rcf for **00:03:00 min** at **4 °C** to ensure nuclei are properly suspended in solution.

Volume added, even by sorting 100 nuclei is minimal in our hands and does not require concentration adjustments.

Transposase Denaturation

8 **Transposase Denaturation**

Denature remaining transposase in sorted nuclei using SDS mixture on Eppendorf Thermocyclers.

55 °C for **00:20:00 min**

96-plex PCR

9 **Amplifying single cell libraries**

Nextera PCR Mater Mix currently produces the highest quality libraries. An alternative master mix using Kapa Hifi Non-Hotstart has been developed and produces good results.

step case

Using Nextera PCR Master Mix

10 Add 13.5 uL PCR Master Mix to each well

7.5 µl NPM

0.25 µl 100X SYBR Green I

5.5 µl dH2O

Perform Real-time PCR on the Bio-Rad CFX Connect:

Temperature (C)	Time	
72	5 min	
98	30 sec	
98	30 s	
55	30 s	
72	1 min	x30 Cycles
	PLATE READ	
72	20 sec	

PCR protocol for Kapa Hifi Non-Hotstart Library Amplification

Pull once majority of well begin to plateau. Sci-ATAC libraries amplify between 14-22 cycles dependent on nuclei per well.

Store libraries at **4 °C for 6 months** or **-20 °C forever**

Library Clean-up and Quantification

11 **Pool post-PCR Product**

Pool 10 uL from each well into 15mL conical tube.

12 **Concentrate DNA via column clean up**

Run full pool volume through Qiaquick PCR purification column following manufacturer's protocol.

Elute in **50 µl 10 mM Tris-HCl pH 8.0**

13 **Clean by size selection with SPRI beads**

Perform a 1X SPRI bead size selection (selecting for DNA > 200 bp).

Add **50 µl 18% PEG SPRI Beads** to column elution, once beads are at room temperature.
Let mixture incubate at room temperature for **00:05:00 min**

Place tube on magnetic rack and wait for magnetic beads to pellet and elution to fully clear (roughly **00:02:00 min**)
Remove full volume of elution without disrupting bead pellet.

Resuspend bead pellet in freshly prepared **100 µl 80% ethanol (v/v)**
Remove full volume of elution without disrupting bead pellet.
Let beads fully air dry (roughly **00:08:00 min**)

- Beads will first lose sheen, and then begin to form cracks.

Resuspend beads off magnetic rack in **31 µl 10 mM Tris-HCl pH 8.0**
Let mixture incubate at room temperature for **00:05:00 min** for DNA to fully become suspended.

Place tube on magnetic rack and wait for magnetic beads to pellet and elution to fully clear (roughly **00:20:00 min**)
Remove full volume of elution without disrupting bead pellet and move to clean tube.

14 **Qubit DNA HS Quantification**

Quantify DNA concentration with 1uL eluted sample on Qubit DNA High-sensitivity kit following manufacturer's protocol.

15 **Agilent DNA HS Bioanalyzer Quantification**

Dilute sample to 4 ng/uL based on read out of Qubit by addition of 10mM Tris-HCl pH 8.0.
Run 1 uL sample on Agilent DNA HS Bioanalyzer following manufacturer's protocol.

Sequencing

16 **Custom Nextseq500 Chemistry Protocol**

Custom primers and sequencing protocol for sci-atac libraries.

96-plex PCR

step case

Using Kapa Hifi Non-Hotstart

10 Add 13.5 uL PCR Master Mix to each well

- **5 µl 5X Kapa Hifi Buffer (GC Buffer)**
- **0.75 µl 10 mM Kapa dNTP Mix**
- **0.5 µl 1 U/uL Kapa Hifi DNA Polymerase**
- **0.25 µl 100X SYBR Green I**
- **6 µl dH2O**

Perform Real-time PCR on the Bio-Rad CFX Connect:

Temperature (C)	Time	
72	5 min	
98	30 sec	
98	30 s	
55	30 s	
72	1 min	x30 Cycles
	PLATE READ	
72	20 sec	

PCR protocol for Kapa Hifi Non-Hotstart Library Amplification

Pull once majority of well begin to plateau. Sci-ATAC libraries amplify between 14-22 cycles dependent on nuclei per well.

Store libraries at **4 °C for 6 months** or **-20 °C forever**

Non-hotstart polymerase is critical for this. As persisting gaps in the genomic DNA have to be filled prior to amplification. This is done by the polymerase at lower temperature (72C for 5 min) prior to the actual PCR.

Library Clean-up and Quantification

11 **Pool post-PCR Product**

Pool 10 uL from each well into 15mL conical tube.

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Sequencing

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Custom primers and sequencing protocol for sci-atac libraries.
To be expanded.