

Oct 15, 2025

scFFPE-ATAC for high-throughput single cell chromatin accessibility profiling in formalin-fixed paraffin-embedded samples

 [Nature Communications](#)

DOI

<https://dx.doi.org/10.17504/protocols.io.4r3l21oq3g1y/v1>

Ram Prakash Yadav¹, Pengwei Xing¹, xingqi chen¹

¹Department of Immunology, Genetics and Pathology, Uppsala University, Uppsala, Sweden



xingqi chen

Uppsala University

Create & collaborate more with a free account

Edit and publish protocols, collaborate in communities, share insights through comments, and track progress with run records.

Create free account

OPEN  ACCESS



DOI: <https://dx.doi.org/10.17504/protocols.io.4r3l21oq3g1y/v1>

External link: <https://doi.org/10.1038/s41467-025-66170-4>

Protocol Citation: Ram Prakash Yadav, Pengwei Xing, xingqi chen 2025. scFFPE-ATAC for high-throughput single cell chromatin accessibility profiling in formalin-fixed paraffin-embedded samples. **protocols.io**

<https://dx.doi.org/10.17504/protocols.io.4r3l21oq3g1y/v1>

**Manuscript citation:**

Yadav RP, Xing P, Zhao M, Hollander P, Strell C, Xie M, Salehi M, Torell E, Sjöblom T, Enblad G, Amini R, Swartling FJ, Glimelius I, Micke P, Hellström M, Chen X (2025) scFFPE-ATAC enables high-throughput single cell chromatin accessibility profiling in formalin-fixed paraffin-embedded samples. Nature Communications 16(). doi: [10.1038/s41467-025-66170-4](https://doi.org/10.1038/s41467-025-66170-4)

License: This is an open access protocol distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Protocol status: Working

We use this protocol and it's working

Created: July 30, 2025

Last Modified: October 15, 2025

Protocol Integer ID: 223667

Keywords: single cell chromatin accessibility, FFPE samples , possible single cell chromatin profiling of ffpe, throughput single cell chromatin accessibility profiling, possible single cell chromatin profiling, cell chromatin accessibility profiling technology, chromatin accessibility profiling technology, chromatin accessibility profiling, cell chromatin accessibility, specific epigenetic profiles in ffpe sample, chromatin accessibility, mediated dna damage rescue, specific epigenetic profile, dna damage rescue, throughput dna barcoding, engineered ffpe, transcription factor, interaction between transcription factor, extensive dna damage, ffpe, ffpe sample, transcription, gene expression, single cell, t7 promoter, dna, gene

Abstract

Formalin-fixed paraffin-embedded (FFPE) samples are the gold standard for tissue preservation in both clinical practice and biomedical research. Chromatin accessibility governs gene expression by modulating the interaction between transcription factors and DNA. Current single-cell chromatin accessibility profiling technology fails to resolve cell-type-specific epigenetic profiles in FFPE samples due to extensive DNA damage. To address this technological gap, we introduce scFFPE-ATAC, an innovative, high-throughput single-cell chromatin accessibility assay for FFPE samples. scFFPE-ATAC integrates a newly engineered FFPE-Tn5 transposase, ultra-high-throughput DNA barcoding (>56 million cell barcodes per run), T7 promoter-mediated DNA damage rescue, and *in vitro* transcription. Our simple step-by-step workflow makes possible single cell chromatin profiling of FFPE archived biological samples.

Materials

Supplementary Table 1: Supplementary_Table1.xlsx 372.1KB

1. Mouse spleen FFPE tissue blocks (Produced in local lab following standard proposal)
2. Xylene (Histolab, cat. no. 2070)
3. Ethanol (VWR BDH Chemicals, cat. no: VWRC20816.552)
4. Collagenase (Sigma-Aldrich, cat. no. C9263)
5. Hyaluronidase (Sigma-Aldrich, cat. no. HX0514)
6. Homogenization buffer (250 mM sucrose, 25 mM KCl, 5 mM MgCl₂, 20 mM Tricine-KOH pH 7.8, 1 mM DTT, 0.5 mM spermidine, 0.15 mM spermine, 0.3% IGEPAL CA-630, complete Protease Inhibitor Cocktail and 0.1% Triton X-100)
7. Ampicillin (Serva, cat. No: 69-52-3)
8. Sodium azide (Merck Millipore, cat. no. 822335)
9. Dounce homogenizer (Merck, cat. no. D9938)
10. MACS® SmartStrainers (Miltenyi Biotech MACS , cat. no. 130-098-458)
11. pluriStrainer Mini (pluriSelect, cat. 43-10020-50 and 43-10010-50)
12. RNase A (Thermo Fisher Scientific, cat. no. EN0531)
13. 1.5 mL LoBind tube (Sarstedt, cat. no: 72.706.600)
14. RSB-T buffer (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 3 mM MgCl₂, 0.1% Tween-20)
15. Tn5 transposase production reagents (pTXB1-Tn5 plasmid, Addgene, 60240)
16. Iodixanol solution (Optiprep, Sigma-D1556)
17. Lysis buffer (20 mM HEPES-KOH pH 7.2, 0.8 M NaCl, 1 mM EDTA, 10% glycerol, 0.2% Triton X-100, complete protease inhibitor)
18. Complete Protease Inhibitor Cocktail (Sigma-Aldrich, cat. no. 11873580001)
19. Dialysis buffer (100 mM HEPES-KOH pH 7.2, 0.2 M NaCl, 0.2 mM EDTA, 2 mM DTT, 0.2% Triton X-100, 20% glycerol)
20. 1× PBS (pH = 7.4) (Thermo Fisher Scientific, cat. no. 10010023)
21. Costar spin-X centrifuge (Coster, cat. no. 8162)
22. 15 mL DNA LoBind tube (Eppendorf, cat. no. 0030122208)
23. Oligo resuspension buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA)
24. Applied Biosystems Veriti 96-Well thermal cycler (Applied Biosystems, cat. no. A27926)
25. Nuclease-free water (Invitrogen, cat. no. AM9932)
26. NEBuffer 3.1 (NEB, cat. no. B7203)
27. T4 DNA ligase (NEB, cat. no. M0202L)
28. MicroAmp fast 96-well reaction plate (0.1ml) (Applied Biosystems, cat. no. 148024Q11G)
29. BSA (Miltenyi Biotech MACS, cat. no. 130-091-376)
30. IGEPAL CA-630 (Sigma-Aldrich, cat. no. I3021)
31. 1 M CaCl₂ (Alfa Aesar, cat. no. J63122)
32. 5 M NaCl (Thermo Fisher Scientific, Invitrogen, cat. no. AM9759)
33. 1 M Tris-HCl pH 8.0 (Thermo Fisher Scientific, Invitrogen, cat. no. 15568025)
34. 1 M Tris-HCl pH 7.5 (Thermo Fisher Scientific, Invitrogen, cat. no. 15567027)
35. 1 M MgCl₂ (Thermo Fisher Scientific, Invitrogen, cat. no. AM9530G)

36. FBS (Life Technologies, cat. no. 10108-105)
37. RNase Inhibitor (Thermo Fisher Scientific, cat. no. N8080119)
38. DTT (Thermo Fisher Scientific, cat. no. A39225)
39. 0.5 M EDTA (Thermo Fisher Scientific, Invitrogen, cat. no. AM9260G)
40. Dimethylformamide (Sigma-Aldrich, cat. no. D4551)
41. Glycerol (Sigma-Aldrich, cat. no. G9012)
42. 10% SDS (Thermo Fisher Scientific, Invitrogen, cat. no. 1553035)
43. Triton X-100 (Sigma-Aldrich, cat. no. T8787)
44. Human genomic DNA (Promega, cat. no. G3041)
45. Qiagen miniElute PCR purification kit (Qiagen, cat. no. 28004)
46. 6× Loading Dye (Thermo Fisher Scientific, cat. no. R0611)
47. HEPES (Sigma-Aldrich, cat. no. H3375)
48. Protease inhibitor (Sigma-Aldrich, cat. no. 11873580001)
49. Spermidine (Sigma-Aldrich, cat. no. S2626)
50. Low Range ssRNA Ladder (NEB, cat. no. N0364S)
51. Proteinase K (Thermo Fisher Scientific, cat. no. EO0491)
52. NEBNext high-fidelity 2× PCR master mix (New England Biolabs, cat. no. M0541S)
53. SPRIselect beads (Beckman Coulter, cat. no. B23317)
54. T7 high yield RNA synthesis kit (New England Biolabs, cat. no. E2040S)
55. Zymo RNA purification kit (Zymo Research, cat. no. R1013)
56. SMART MMLV kit (TAKARA, cat. no. 639524)
57. RNAClean XP beads (Beckman Coulter, cat. no. A63987)
58. Zymo CHIP DNA clean and concentrator kit (Zymo Research, cat. no. D5205)
59. 40% Acrylamide:bis-acrylamide (Invitrogen, cat. no. HC2040)
60. 10% Ammonium persulfate (Invitrogen, cat. no. HC2005)
61. TEMED (Invitrogen, cat. no. HC2006)
62. Tween-20 (Sigma-Aldrich, cat. no. P1379)
63. Nuclease-free water (Invitrogen, cat. no. AM9932)
64. KOH (Sigma-Aldrich, cat. no. 484016)
65. 50 bp DNA ladder (ThermoFisher Scientific, cat. no. 10488099)
66. Agilent high sensitive DNA kit (Agilent, cat. no. 5067-4626)
67. Tn5 transposase, produced in a local protein facility as previously described (Picelli *et al.*, 2014).
68. Oligonucleotides (FFPE_Tn5_DNA_#1-64)
69. 2 mL DNA LoBind tube (Eppendorf, cat. no. 0030108078)
70. Novex™ TBE-Urea Gels, 10%(Invitrogen, cat. no. EC6875BOX)
71. Spermine (Millipore-Sigma, cat. no. S3256)
72. 2 M KCl (Thermo Fisher Scientific, cat. no. AM9640G)
73. Trypan Blue Solution 0.4% (Thermo Fisher Scientific, cat. no. 15250061)
74. Sucrose (Millipore-Sigma, cat. no. S7903)
75. Tricine (Millipore-Sigma, cat. no. T0377)
76. Potassium Hydroxide (Millipore-Sigma, cat. no. P5958)
77. RNase H (Thermo Fisher Scientific, cat. no. EN0201)
78. 1 mL syringe (BD Microlance, cat. no. 309628)



79. 21G needle (BD Microlance, cat. no. 302200)

80. Parafilm (Merck, cat. no. HS234526A)

81. All DNA oligos were synthesized in Integrated DNA technologies (IDT) (DNA Sequences are provided in Supplementary Table 1)

References:

- (1) Picelli, Simone, et al. "Tn5 transposase and tagmentation procedures for massively scaled sequencing projects." *Genome research* 24.12 (2014): 2033-2040.
- (2) Grandi, Fiorella C., et al. "Chromatin accessibility profiling by ATAC-seq." *Nature protocols* 17.6 (2022): 1518-1552.
- (3) Yadav, Ram Prakash, et al. "FFPE-ATAC: A highly sensitive method for profiling chromatin accessibility in formalin-fixed paraffin-embedded samples." *Current protocols* 2.8 (2022): e535.

Safety warnings

- ⚠ Deparaffinization involves the use of xylene, a toxic aromatic solvent. This step must be carried out in a fume hood to ensure proper ventilation and user safety. It is recommended to use a fume hood and wear appropriate personal protective equipment (PPE), including a lab coat, gloves, and eye protection, while preparing any stock solutions with toxic chemicals such as dithiothreitol (DTT), sodium azide, ampicillin, dimethylformamide (DMF), etc. While the remaining steps of the protocol do not involve hazardous chemicals or dangerous equipment, standard laboratory safety training is recommended before starting. Always follow your institution's safety guidelines and wear appropriate personal protective equipment.

REAGENT SETUP

1 REAGENT SETUP

Tn5 transposase production: Tn5 transposase (produced in local protein facility) following previous report (**Ref. 1**). Store at -80°C until use.

Tissue homogenization buffer: 250 mM sucrose, 25 mM KCl, 5 mM MgCl_2 , 20 mM Tricine-KOH pH 7.8, 1 mM DTT, 0.5 mM spermidine, 0.15 mM spermine, 0.3% IGEPAL CA-630 and complete Protease Inhibitor Cocktail. In addition, add 0.1% Triton X-100 in the tissue homogenization buffer for mouse spleen, human lymph node, and human lymphoma samples. Always prepare fresh just before the experiment.

Diluent buffer: 150 mM KCl, 30mM MgCl_2 and 120 mM Tricine-KOH, pH 7.8. Store at 4°C for 1 year.

Iodixanol solutions: Using a 60% iodixanol solution (Optiprep, Sigma-D1556), prepare a fresh 50% iodixanol solution by diluting it with a diluent buffer. Additionally, prepare fresh 36% (or 33.6%) and 48% iodixanol solutions by diluting with a tissue homogenization buffer. Mix gently and keep on ice.

RSB-T buffer: Prepare RSB buffer (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 3 mM MgCl_2) and store at 4°C . Always prepare fresh RSB-T buffer by adding 0.1% Tween-20 in the RSB buffer.

2× Dialysis buffer: 100 mM HEPES-KOH pH 7.2, 0.2 M NaCl, 0.2 mM EDTA, 2 mM DTT, 0.2% Triton X-100, and 20% glycerol. Store at -20°C until use.

Note: Dithiothreitol (DTT) is toxic; always remember to wear personal protective equipment such as gloves, eye protection, and a lab coat while preparing reagent in a fume hood.

Lysis buffer: 10 mM Tris-Cl, pH 7.4, 10 mM NaCl, 3 mM MgCl_2 , and 0.1% IGEPAL CA-630. Always prepare fresh just before the experiment.

2× Tagmentation buffer: 20 mM Tris-HCl, pH 7.5, 10 mM MgCl_2 , 20% dimethylformamide, and ddH_2O . Store at -20°C until use.

Note: Dimethylformamide (DMF) is toxic. Use only under a chemical fume hood. Wear personal protective equipment such as gloves, eye protection, and a lab coat while preparing reagent in a fume hood.



Pre-coat 15 mL tube with 0.5% BSA: Add 500 μ L of 10% BSA (Miltényi Biotech MACS, cat. no. 130-091-376) in the 9.5 ml sterilized water, mix, and filter with a 0.22 μ M Millex GP Filter Unit (cat. no. SLGP033RB). Then, transfer 0.5% BSA solution into a 15 ml DNA LoBind tube (Eppendorf, cat. no. 0030122208) and rotate slowly at room temperature for 1-2 hours before using it.

2 x Reverse cross-linking buffer: 100 mM Tris-HCl, pH 8, 500 mM NaCl, 2 mM EDTA, and 2% SDS. Store at -20°C until use.

Recipe of 6% Polyacrylamide gel (one PAGE gel)

	A	B
	Reagents	Volume (10 mL)
	40% Acrylamide:bis-acrylamide (29:1)	1.5 mL
	Sterile Water	7.44 mL
	10 $\times$ TBE buffer	1 mL
	10% Ammonium persulfate	50 μ L
	TEMED	10 μ L

Note: PAGE gel-preparing reagents (such as acrylamide:bisacrylamide, ammonium persulfate, and TEMED) are toxic chemicals, so we always recommend safely handling these chemicals, wearing appropriate personal protective equipment (PPE) such as gloves, eye protection, and a lab coat, and preparing the gel in a fume hood.

Crush-soak buffer: 500 mM NaCl, 1 mM EDTA, and 0.5% SDS. Store at room temperature.

Oligo ramping for scFFPE-ATAC: Prepare three DNA barcoded plates by ramping the barcodes (Ligation 1_#1-96, Ligation 2_#1-96, and Ligation 3_#1-96) with respective linkers (Linker-Ligation 1, Linker-Ligation 2, and Linker-Ligation 3) oligos in a 96-well plate. For 10 μ L of total volume, add 1.2 μ L of 100 μ M barcode plus 8.8 μ L of 12.5 μ M linker oligos per well and mix it. Seal the plates and anneal in a thermocycler with the following program: 95°C for 5 min, then slow cooling to 20°C with a temperature ramp of -0.1°C/s .

Note: For large sets of experiments, we suggest you scale the respective barcode and linker to 50 μ L volume. Then, after ramping, split 10 μ L per well with multichannel pipette

and properly seal the MicroAmp™ Fast Optical 96-Well Reaction Plate (Applied Biosystems, cat. no. 4346907) with sealing tape (Sarstedt, cat. no. 95.1994). The ramped oligos can be stored at -20°C for 6-12 months.

FFPE-Tn5 transposase assembly: Resuspend oligonucleotides (FFPE-Tn5 DNA #1-64) in oligo resuspension buffer (10 mM Tris-HCl pH 8.5, 0.1 mM EDTA) to a final concentration of 100 μM each. Mix equimolar amounts of MEbottom blocked and FFPE-Tn5 DNA #1-64 in separate 200 μL PCR tubes. Anneal adaptors on the PCR machine with the following PCR program: 95°C for 5 min, then ramp down to 20°C at $-0.1^{\circ}\text{C}/\text{s}$ (hold at 20°C for 5 min). Assemble Tn5 transposase with the following components: 4 μM of ME bottom blocked FFPE-Tn5 DNA ramped hybrid oligonucleotides, 40% of 100% glycerol, 0.61 $\times$ of 2 $\times$ dialysis buffer, and 2 μM of pure Tn5. Make up the reaction mixture volume to 50 μL by adding nuclease-free water (Invitrogen, AM9932), gently mix, and incubate for 1 hour at 25°C for annealing of oligos to Tn5. The loaded Tn5 transposase with its adaptors can be stored at -20°C for 1-3 months.

Note: We recommend verifying the activity of FFPE-Tn5 transposases (#1-64) prior to conducting the scFFPE ATAC to ensure its optimal performance. To perform *in vitro* FFPE-Tn5 transposase activity assay, mix the following reagents: 10 μL of 2 $\times$ TD buffer, 50 ng of human genomic DNA (Promega, cat. no. G3041), 1 μL of assembled FFPE-Tn5 (2 μM), and make up to 20 μL using sterile water. Mix and incubate the reaction mixture at 55°C for 7 minutes, then directly purify it using the Qiagen MinElute PCR Purification kit according to the manufacturer's instructions. Elute the purified samples in 10 μL of elution buffer. Mix the samples with 2 μL of 6 $\times$ loading dye (Thermo Fisher Scientific, cat. no. R0611). Then, run samples on a 1% agarose gel (see Figure 1) to check the size of the tagged DNA using a 50 bp DNA ladder (Invitrogen, cat. No. 10416014) as a reference.

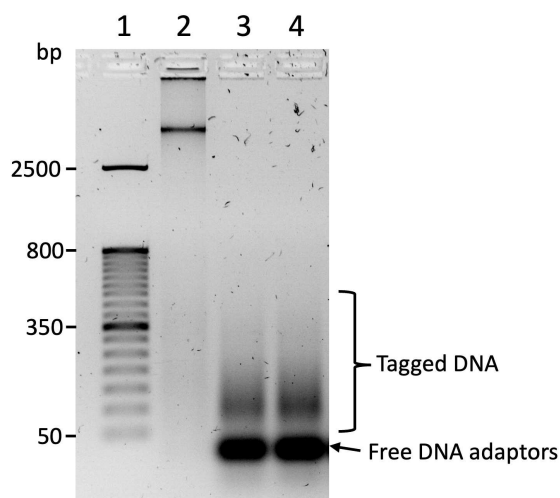


Figure 1: 1% agarose gel analysis showing samples (1-DNA ladder, 2-input 50 ng of human genomic DNA, 3-4-tagged DNA samples).

Tn5 transposase activity quantification: The final concentration of Tn5 transposase in our study was determined following a previous report (**Ref. 2 and 3**). A brief description of the process is as follows: The homemade Tn5 was diluted with the dilution buffer (50 mM Tris, 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1% NP-40, and 50% glycerol) at different concentrations. Tagmentation was performed on 50 ng of purified genomic DNA instead of cells. We quantified the number of cycles required to reach one-third of the plateau fluorescence by qPCR and determined the final dilution factor of homemade Tn5 that showed the most similar number of cycles as Nextera TDE1.

Nuclei isolation from FFPE tissues

- 2 To obtain intact nuclei, rather than fragmented nuclei with chromatin leakage, the tissue section thickness should be greater than 10 μm . Take a 1–1.2 mm punch core from the FFPE tissue block, or cut a 20–50 μm section FFPE tissue block (see **Figure 2**). Proceed with deparaffinization by placing a puncture or section into 1 mL of xylene (three times), followed by 1 mL of ethanol (100% EtOH; twice) for 5 mins.

Note: Xylene is a toxic aromatic solvent. Therefore, it is recommended to handle this chemical safely in a fume hood. Always wear appropriate personal protective equipment (PPE), including gloves, eye protection, and a lab coat, while performing deparaffinization.

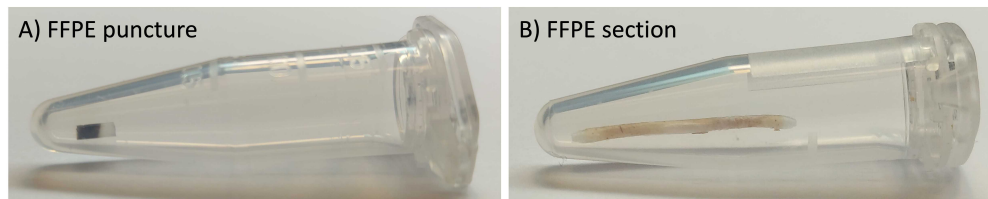


Figure 2. A tissue puncture and section derived from FFPE tissue blocks in the Eppendorf tubes.

Subsequently incubate the sample in a sequential rehydration series in 1 mL of 95%, 70%, 50%, and 30% EtOH, followed by incubation in sterilized water and PBS (pH 7.4) for 5 minutes each.

Note: While performing the deparaffinization and sequential rehydration series, small thin sections or tiny pieces of tissue samples may float in the liquid. It is likely that we lose samples. Therefore, it is advisable to avoid using a large-volume pipette. Instead, we recommend using a small-volume pipette (e.g., 200 μL) to slowly and carefully remove the liquid, ensuring that the samples are not lost.

BREAK POINT: After deparaffinization and rehydration for large sets of samples, it is possible to keep the samples in 200–500 μ l of 1x PBS (pH: 7.4) containing 50 μ g/ml sodium azide. Seal the tube tightly with Parafilm (Merck, cat. no. HS234526A) and keep it at 4°C. We recommended processing the samples as soon as possible, within 1–3 days.

- 3 Perform tissue microdissection to obtain fine tissue pieces using a sharp blade (see **Figure 3**) and enzymatically digest the tissue with a collagenase (3 mg/mL) and hyaluronidase (1 mg/mL) cocktail mix (1 mL) in PBS containing 0.5 mM CaCl_2 , 50 μ g/ml sodium azide, and 100 μ g/ml ampicillin at 37°C for 4 hours at 850 rpm.

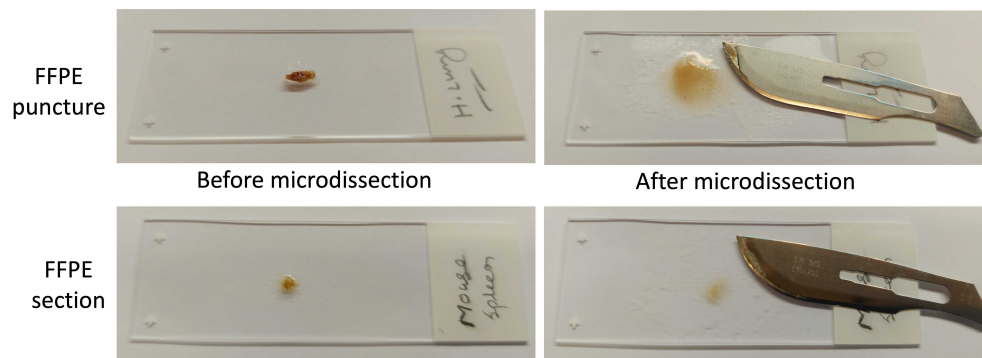


Figure 3. Microdissection of the FFPE tissue puncture and section on the microscope slide using a sharp blade.

Note: This step is crucial, so we suggest allocating at least 10–20 minutes for each sample. Begin slicing and making smaller, finer pieces of tissue using Sterile Stainless Steel Scalpel Blades (cat. no. 12941645, Fischer Scientific) on the microscope slide (25×75×1 mm, cat. no. J1800AMNZ, Epredia) (see **Figure 3**), which can be easily collected by directly placing 200–500 μ l of enzyme mix onto the slides and tilting the enzyme mix suspension containing microdissected tissue towards the corner of the slide facing towards the opening of 2-mL Lo-bind tubes (Sarstedt, cat. no. 72.695.600) and partially digesting the microdissected tissue in 1 mL of enzyme cocktail for the 20 μ m of tissue sections (1–2) or for a tissue puncture measuring 1–1.2 mm. If you intend to process additional tissue sections or a larger puncture per sample, we recommend adjusting the enzyme volume accordingly.

The duration of partial enzymatic digestion required to achieve a good yield of nuclei depends on the type of tissue being processed. For soft tissues (e.g., mouse spleen and human lymph nodes), normally 4 hrs is enough, whereas solid tumors (e.g., pancreatic ductal adenocarcinoma (PDAC)) need more time (over 4 hours) due to their high extracellular matrix content.

To determine the exact enzymatic digestion time for different tissues, a good approach is to perform a time-series digestion experiment. It is always useful to check the digestion efficiency by examining the sample under a microscope with nuclei staining (see **Figure 4**), which shows examples of incomplete and complete tissue digestion from human lung tumors. It is important to add sodium azide and ampicillin to prevent bacterial growth during enzyme digestion.

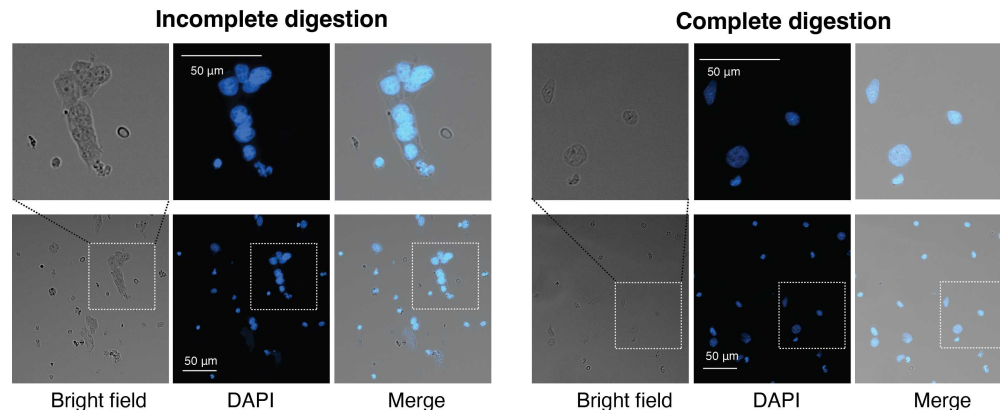


Figure 4. The microscopy images depict both complete and incomplete enzymatic digestion of FFPE human lung tumor.

- 4 Wash the sample twice with 1 mL PBS by centrifuging 1500 g for 5 min at 4°C.



BREAK POINT: After enzymatic digestion and washes, it is possible to keep the samples in 200–500 µl of 1x PBS (pH: 7.4) containing 50 µg/ml sodium azide. Seal the tube tightly with Parafilm (Merck) and keep it at 4°C. We recommended processing the samples as soon as possible within 1–2 days.

- 5 Add ice-cold 1 mL tissue homogenization buffer. Gently pipette up and down and incubate on ice for 20–30 minutes for tissue lysis. Transfer the tissue containing homogenization buffer in a Dounce homogenizer (Merck, cat. no. D9938) placed in ice and homogenize the mixture with 10 strokes using a loose pestle, filter through a 30 µm filter (MACS® SmartStrainers, cat. no. 130-098-458), then subject to another 10 strokes using a tight pestle and filter sequentially through 20 µm and 10 µm filters (pluriStrainer Mini, cat. no. 43-10020-50 and 43-10010-50).

Note: We performed the protocol using different samples derived from mouse spleens, human lymph nodes, human lymphoma and human lung cancers. Comparing such diverse tissues, we found in the case of human lung cancer that tissue homogenization buffer without 0.1% Triton X-100 for tissue lysis and without loose and tight dounce homogenization releases less extracellular matrix and cellular debris, which ultimately

minimizes the blockages in the filter during the subsequent filtration process. So for human lung cancers, we recommend avoiding Triton X-100 in the tissue homogenization buffer for tissue lysis. After 20–30 minutes of incubation on ice, mix the sample by pipetting up and down (10–20 times) and directly filter through 30 μm , 20 μm , and 10 μm filters without loose and tight dounce homogenization.

Sometimes, filtration gets halted if there are more concentrated input nuclei with dense extracellular matrices; in that case, we suggest diluting the sample and splitting the same samples into 2–3 filters. Pre-wetting the filters with homogenization buffer before performing filtration is critical to get a good yield of nuclei.

- 6 Following filtration, add 0.1% RNase A (Thermo Fisher Scientific, cat. no. EN0531) and mix gently and incubate at room temperature (RT) for 5 minutes. Centrifuge the samples at 400 g for mouse spleen and human lymph node samples and 1000 g for human lung samples at 4°C for 5 minutes to obtain nuclei pellets. Remove the supernatant and resuspend in 300 μL of fresh homogenization buffer.
- 7 Perform density gradient centrifugation to remove cell debris and extracellular matrices from the single-cell nuclei suspension. Mix the nuclei suspension in a 1:1 ratio with a 50% iodixanol solution to create a 25% gradient mix, resulting in a total volume of 600 μL of nuclei. Load this mixture onto the top layer of the density gradient containing 600 μL per density gradient mix: 36% and 48% for mouse spleen and human lymph node (see **Figure 5**), and 33.6% and 48% for human lung cancer, respectively, in a 2 mL DNA LoBind tube (Eppendorf, cat. no. 0030108078). Centrifuge the tubes containing the gradient layers at 3,000 g for 20 minutes at 4°C in a pre-chilled swinging bucket centrifuge.

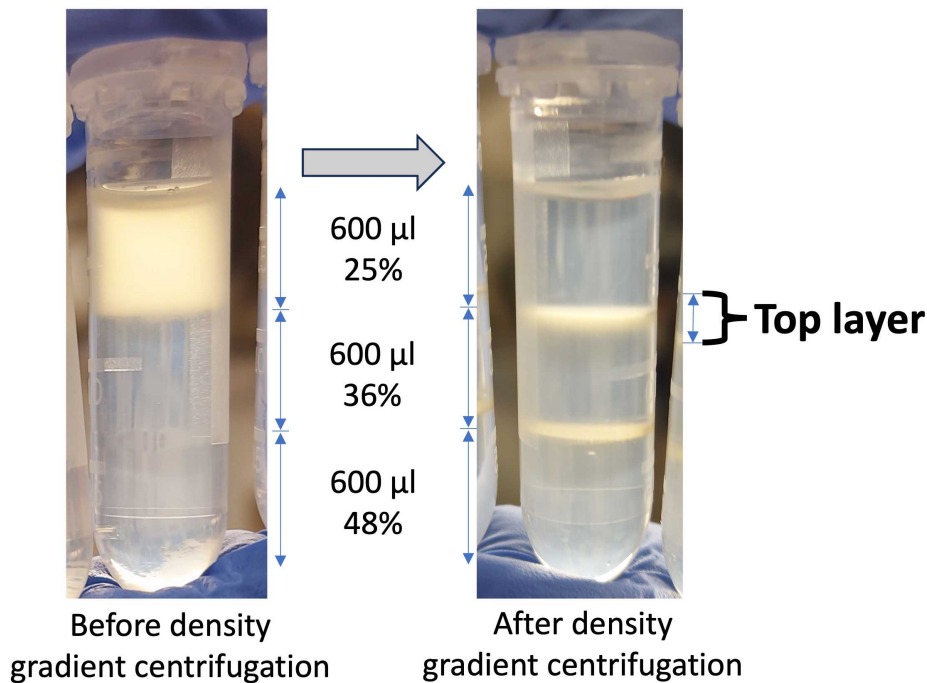


Figure 5. Purification of FFPE mouse spleen nuclei suspension by density gradient centrifugation.

Note: While preparing the density gradient, always remember not to shake or intermix the density gradient layers; otherwise, the top and bottom layers will disappear after density gradient centrifugation. We recommend first placing a 25% gradient mix that contains nuclei suspension in the bottom of the 2 mL DNA LoBind tube. Then, slowly push the 25% gradient mix from the bottom by releasing the 36% (or 33.6%) gradient mix from the 1 mL syringe (BD Microlance, cat. no. 309628) fitted with a 21G needle (BD Microlance, cat. no. 302200). Follow the procedure by similarly releasing 48% of the gradient mix at the bottom of the tube.

- 8 After centrifugation, collect 300 µL of the nuclei band visible at the 25%-36% (or 25%-33.6% for human lung tissue in our hand) interface (top layer, see **Figure 5**) and transfer to a 1.5 mL LoBind tube (Sarstedt).

Note: If you don't see a dense white band on the top layer, it means either you have less input or a lower number of nuclei after density gradient centrifugation. Then just carefully mark the 25%-36% (or 25%-33.6%) interface with a marker pen and collect the 300-400 µL from the top layer without shaking.

- 9 Dilute the nuclei suspension by adding an equal volume (300 µL) of RSB-T buffer. Gently mix by pipetting and centrifuge at 600 g for 5 minutes to collect the nuclei. Resuspend the nuclei in 200-500 µL of PBS containing sodium azide (50 µg/mL) and store at 4°C.

The nuclei suspension is mixed with Trypan blue stain (Invitrogen, cat. no. T10282) in a 1:1 ratio, using a volume of 10 μL , and then loaded into the Cell Counting Chamber Slide (Invitrogen, cat. no. C10283) for counting nuclei using the Cell Counter.

After density gradient centrifugation, nuclei quality is assessed under the microscope by staining them with DAPI and preparing a microscopy slide. Example images without and with density gradient centrifugation are shown in **Figure 6**.

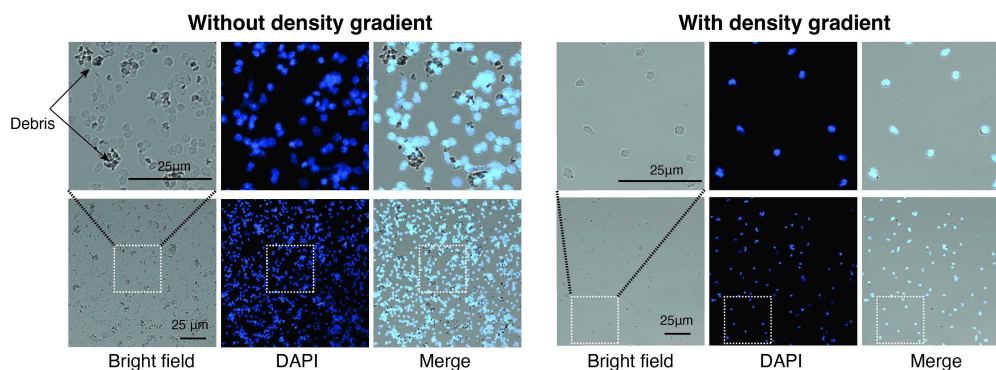


Figure 6: The examples of microscopy imaging without and with density gradient centrifugation of FFPE mouse spleen nuclei.

BREAK POINT: After nuclei isolation for large sets of samples, it is possible to keep the nuclei in 200–500 μL of 1x PBS (pH: 7.4) containing 50 $\mu\text{g}/\text{mL}$ sodium azide. Seal the tube tightly with Parafilm (Merck) and keep it at 4°C. We recommended processing the samples as soon as possible within 1–3 days.

Tagmentation and combinatorial indexing via ligation

- 10 Before tagmentation, centrifuge 50,000 purified FFPE single-cell nuclei at 2,000 g for 5 minutes at 4°C and resuspend in 50 μL lysis buffer. Centrifuge the nuclei again at 2,000 g for 5–10 minutes at 4°C. Resuspend the nuclei in 95 μL of 1x tagmentation buffer, and add 5 μL of barcoded FFPE-Tn5 transposase (2 μM) per sample. Gently mix and incubate at 37°C for 30 minutes at 400 rpm.

Note: For a large number of tagmentation reactions, we recommend conducting the reactions using 50,000 nuclei in 96-well plates, with volumes of either 50 μL or 100 μL , while ensuring a consistent enzyme concentration throughout.

- 11 After tagmentation, place the tagmentation reactions on ice and stop the reaction by adding an equal volume (100 μL) of 50–60 mM EDTA to each sample. Pool the samples

into a 15 mL DNA LoBind tube (Eppendorf) pre-coated with 0.5% BSA, followed by centrifugation at 1,000 g for 10 minutes at 4°C in a swing centrifuge.

- 12 Perform three rounds of ligation using the split-and-pool technique in a 96-well plate, with each well containing a ramped specific linker and ligation barcode. Resuspend the pooled nuclei in 1 mL of 1× NEBuffer 3.1 and mix with the ligation mix (100 µL of 10× NEBuffer 3.1, 22 µL of 50 mg/mL BSA, 500 µL of 10× T4 DNA ligase buffer, 2,278 µL of ultrapure water, and 100 µL of T4 DNA ligase).

Transfer 40 µL of this mix per well, containing 10 µL of the ramped Ligation1 mixture, mix and incubate at 37°C for 30 minutes at 400 rpm. Add 10 µL of Blocker-Ligation1 solution (2.64 µL of 100 µM Blocker-Ligation1, 2.50 µL of 10× T4 ligation buffer, and 4.86 µL of ultrapure water) per well, mix and incubate at 37°C for 20 minutes at 400 rpm.

- 13 Pool the samples into a 15 mL DNA LoBind tube (Eppendorf) pre-coated with 0.5% BSA, followed by centrifugation at 1,500 g for 10 minutes at 4°C in a swing centrifuge.

- 14 The second and third ligation reactions are performed identically to the first. After the second and third ligations, blocking reactions are carried out using 10 µL of Blocker-Ligation2 solution per well (2.64 µL of 100 µM Blocker-Ligation 2, 50 µL of 10× T4 ligation buffer, and 4.86 µL of ultrapure water) and 7.5 µL of TerminatorLigation3 solution per well (2.64 µL of 100 µM TerminatorLigation3, 2.50 µL of 0.5 M EDTA, and 2.36 µL of ultrapure water) for reaction termination.

After gently mixing, the samples are pooled again into a 15 mL DNA LoBind tube pre-coated with 0.5% BSA, followed by centrifugation at 1,500 g for 10 minutes at 4°C.

Note: Our scFFPE-ATAC method is effective for processing a minimum of 500,000 to 1 million nuclei. For clinical samples that are limited to fewer than 500,000 nuclei, we recommend increasing the centrifugation speed to between 2,000 and 3,000 g before tagmentation and during ligation steps. This adjustment will help minimize the loss of nuclei.

- 15 After the third ligation, each nucleus acquires a unique combination of barcodes. Finally, remove the supernatant and resuspend in PBS; nuclei are counted and then divided into 30,000 to 80,000 nuclei per tube for the reverse crosslinking.

Reverse-crosslinking, gap filling and *in vitro* transcription

- 16 After the third ligation, add an equal volume of 2x reverse cross-linking buffer and 1 µL proteinase K to each tube, following previous publications. Seal the tube properly with paraffin and incubate the reaction mixture in a thermomixer at 1,200 rpm overnight at 65°C.

17 The next day, perform a second proteinase K digestion by adding 1 μ L of proteinase K at 37°C for 2 hours at 850 rpm to ensure complete protein digestion. Purify DNA using the Zymo ChIP DNA Clean Concentrator Kit (Zymo Research, cat. no. D5205), following the manufacturer's instructions.

18 For gap filling, add an equal volume of NEBNext High-Fidelity 2X PCR Master Mix (New England Biolabs, cat. no. M0541S) to the eluted DNA, and incubate the reaction mixture at 72°C for 8 minutes in a thermal cycler. Purify the sample using the Qiagen MinElute PCR Purification Kit (Qiagen, cat. no. 28004), following the manufacturer's instructions.

BREAK POINT: After purification, it is possible to store samples at -20°C for short-term and -80°C for long-term storage. Avoid repeated freeze-thaw cycles.

19 Further DNA purification is carried out using the 1x SPRIselect beads (Beckman Coulter, cat. no. B23317) following the manufacturer's instructions. Finally, elute the DNA in 13-26 μ L nuclease-free water.

Note: While performing DNA purification with SPRIselect beads, be careful not to overdry the beads. Overdrying can result in longer elution times to retrieve all the DNA or may lead to a partial loss of the sample DNA.

20 For *in vitro* transcription (IVT), use a T7 high yield RNA synthesis kit (New England Biolabs, cat. no. E2040S). Add the following reagents for 25 μ L reaction mix: 12.8 μ L of template DNA, 2.5 μ L of 10X T7 buffer, 1 μ L of DTT, 2 μ L each of ATP, CTP, UTP, and GTP, 1 μ L of T7 mix, and 0.1 μ L of RNase inhibitor (Thermo Fisher Scientific, cat. no. N8080119). Incubate the IVT mixture at 37°C overnight (16 hours).

Note: Due to the high number of nuclei per tube, we conducted the IVT reaction in 50 μ L volume, which is doubling the reaction volume for each sample, and then dividing the reaction mix into two tubes for overnight IVT. After the IVT step, we pool two tubes into one tube for DNase I treatment and RNA purification using the ZYMO RNA Clean & Concentrator-5 Purification Kit (Zymo Research, cat. no. R1013), following the manufacturer's instructions.

21 After IVT, followed by DNase I treatment and RNA purification using the ZYMO RNA Clean & Concentrator-5 Purification Kit (Zymo Research, cat. no. R1013), according to the manufacturer's instructions. Elute RNA in 10-20 μ L of nuclease-free water. Measure the RNA concentration using a NanoDrop.

BREAK POINT: After RNA purification, it is recommended to store samples at -80°C until further use. Avoid repeated freeze-thaw cycles.

22 Load the purified IVT-derived RNAs onto a 10% Novex™ TBE-Urea Gel (Invitrogen, cat. no. EC6875BOX) to assess the size of the RNAs generated during *in vitro* transcription (IVT). We can see a smear of RNAs containing both long and small RNAs (see **Figure 7**)

with the help of Low Range ssRNA Ladder (NEB, cat. no. N0364S). The sample can be used immediately for DNA library preparation or stored at -80°C for future use.

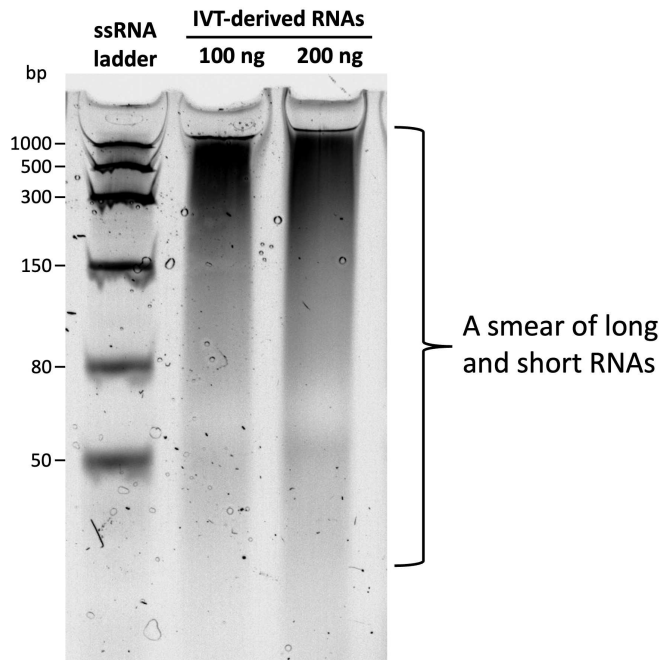


Figure 7. Purified IVT-derived RNAs run in the 10% TBE-Urea Gel.

Single cell FFPE-ATAC DNA library preparation

- 23 Prepare single-stranded cDNAs from 0.5–1 μg of purified IVT transcripts using the FirstStrand_cDNA oligo (DNA oligo sequences are provided in **Supplementary Table 1**) with the SMART MMLV kit (TAKARA, cat. no. 639524). Prepare a reaction mixture (11.25 μL) containing 500 ng–1 μg of RNA, 1 μL of 100 μM FirstStrand_cDNA oligo, and 0.25 μL of RNase Inhibitor (20 U/ μL). Heat at 70°C for 3 minutes, then immediately cool on ice.

Note: *If in vitro transcription yields less than 500 ng of RNA, we recommend using all of it for single-cell DNA library preparation.*

- 24 Next, the master mix is added, which consists of:
 - 4 μL of 5 $\times$ First-Strand Buffer (from SMART MMLV kit (Takara, cat. no. 639524))
 - 2 μL of dNTP Mix (Thermo Fisher Scientific, cat. no. R0192)
 - 2 μL of 100 mM DTT (from SMART MMLV kit (Takara, cat. no. 639524))
 - 0.25 μL of RNase Inhibitor (20 U/ μL) (Thermo Fisher Scientific, cat. no. N8080119)
 - 0.5 μL SMART MMLV Reverse Transcriptase (from SMART MMLV kit (Takara, cat. no. 639524))



The reaction is gently mixed and incubated at 42°C for 60 minutes and then at 70°C for 15 minutes.

- 25 Add 2.2 µL of 10× RNase H buffer and 0.2 µL of RNase H (5 U/µL, Thermo Fisher Scientific, cat. no. EN0201) to each reaction and incubate at 37°C for 20 minutes.
- 26 The RNAClean XP beads (Beckman Coulter, cat. no. A63987) are used (with 1.8x) to purify the cDNA reaction mixture, and the cDNA is eluted in 20 µL of Qiagen Elution Buffer.
- 27 Prepare a sequencing library mix using purified cDNA and add the following reagents: 20 µL of purified cDNA, 25 µL of NEBNext high-fidelity 2× PCR master mix (New England Biolabs, cat. no. M0541S), 4.2 µL of nuclease-free water, 0.4 µL of 10 µM of unique forward (i5), and 0.4 µL of 10 µM of unique reverse (i7) primer combination (DNA oligo sequences are provided in Supplementary Table 1).
- 28 Run PCR amplification with the following cycling program: 98°C for 30 seconds; 12 cycles of: 98°C for 20 seconds, 63°C for 20 seconds, and 72°C for 1 minute.
- 29 Purify the sample using the Qiagen MinElute PCR Purification Kit (Qiagen, cat. no. 28004).

BREAK POINT: After single cell DNA library purification, it is recommended to store samples at -20°C for short-term and -80°C for long-term storage. Avoid repeated freeze-thaw cycles.

- 30 Load and run the samples (For e.g., 1-2: mouse spleen and 3-4: human lymph node) on a 6% PAGE gel containing TBE buffer at 180 V for 37 min.

Note: We recommend loading only half of the sample and keeping the remaining samples as a backup in case any technical issues arise during PAGE gel running.

- 31 Cut the gel region corresponding to 250–800 bp (see **Figure 8**) based on the 50 bp DNA ladder (ThermoFisher Scientific, cat. no. 10488099), and the gel is made into fine pieces by placing the cut gel slice into a 0.5 mL punched tube inside a 2 mL DNA LoBind tube (Eppendorf, cat. no. 0030108078), centrifuging at 16,000 × g for 5 minutes, and discarding the 0.5 mL punched tube.

Note: Punched tubes can be made by making holes in the bottom of a 0.5 mL tube (Invitrogen, cat. no. Q32856) using a 21G needle (BD Microlance, cat. no. 302200).

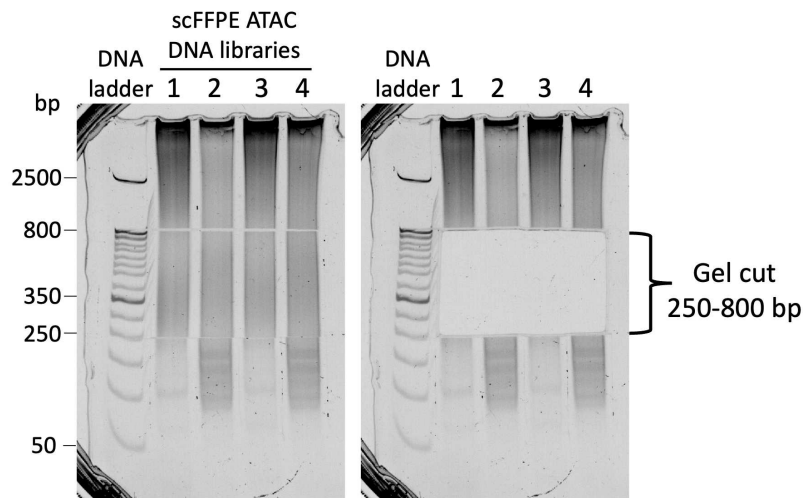


Figure 8. 6% PAGE gel showing a smear of scFFPE ATAC DNA libraries and gel cut size (250-800) bp.

- 32 Incubate the small gel pieces overnight at 55°C in 300 μ L of crush-soak buffer at 1,200 rpm. Purify the DNA from the gel using Costar Spin-X centrifuge tubes (Costar, cat. no. 8162) and the Zymo ChIP DNA Clean Concentrator Kit (Zymo Research, cat. no. D5205), following the manufacturer's instructions.

BREAK POINT: After sequencing library purification, it is recommended to store samples at -20°C for short-term and -80°C for long-term storage. Avoid repeated freeze-thaw cycles.

- 33 Load and run the sequencing DNA libraries in the Biolanalyzer (see **Figure 9**) using the Agilent High Sensitivity DNA Kit (cat. no. AGLS5067-4626) to check the DNA library size distribution and quantify the DNA concentration of the DNA libraries (1-2: mouse spleen and 3-4: human lymph node). Finally, deep sequencing is performed with Illumina sequencer.

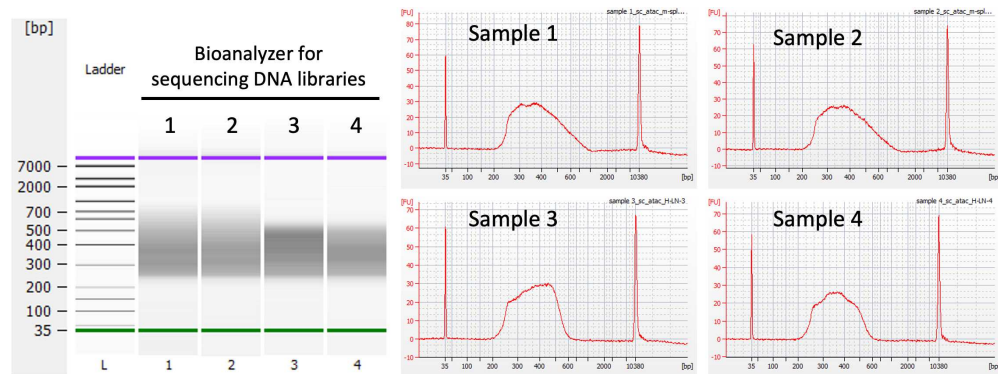


Figure 9. Bioanalyzer showing peak regions of the scFFPE ATAC sequencing libraries.

Demultiplexing split-pooled single cell ATAC-seq data

- 34 A detailed description of our computational pipeline (see **Figure 10**) is provided in the following GitHub link:

<https://github.com/pengweixing/scFFPE>

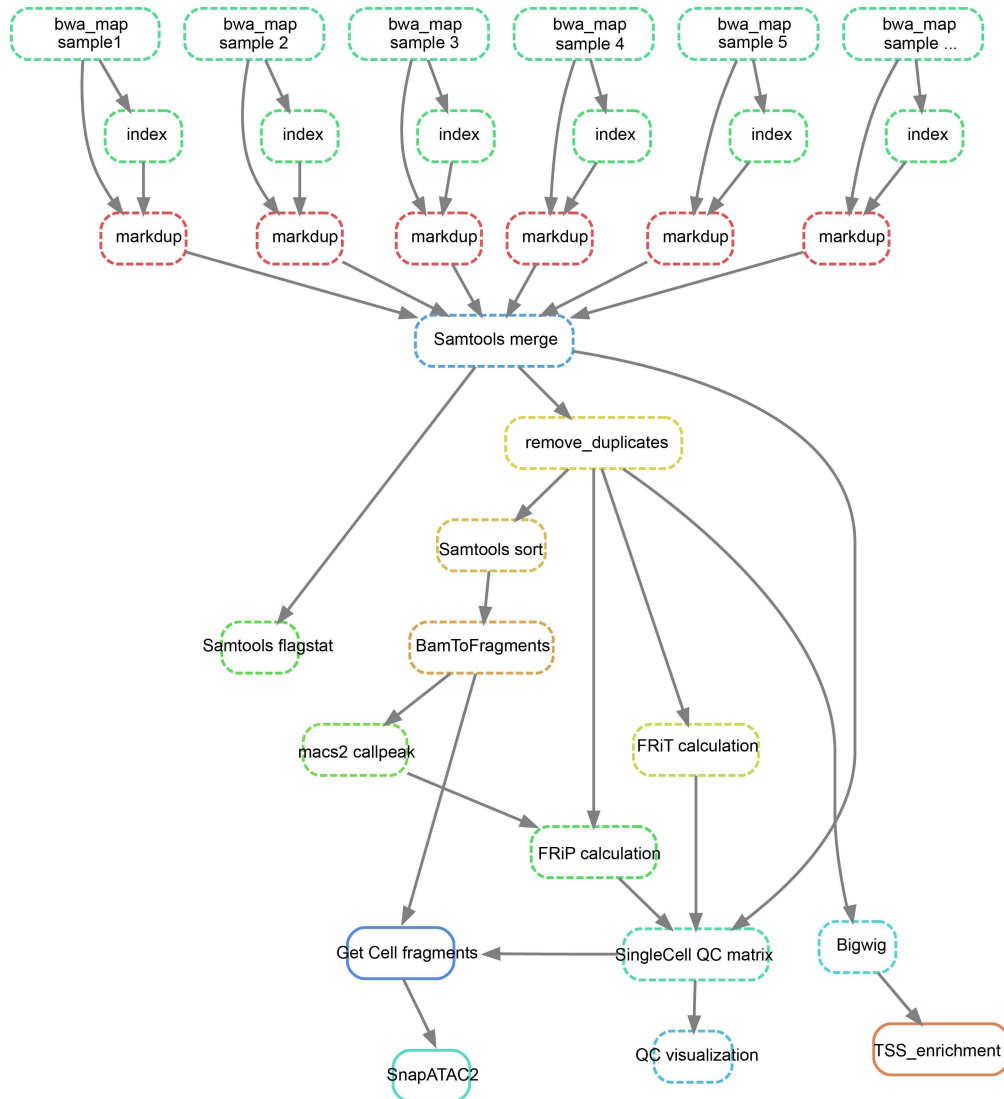


Figure 10: The computational workflow for the single cell decoding, mapping and data analysis

Use an in-house script to demultiplex the split-pool single-cell FFPE-ATAC data and remove adapter sequences from genomic reads (<https://github.com/pengweixing/scFFPE/>). Extract sample-specific Tn5 indices, cellular barcodes, and linker sequences from Read 2. The full barcoding sequence ranges from 88 to 91 bases in length: the Tn5 index is 3 bases long, the cellular barcodes are 7 bases each, and the linker sequence spans 17 bases. The first bases of BC #1, BC #2, BC #3, and the Tn5 index are expected to be located at the 1st, 22–26th, 43–47th, and 66–68th positions of Read 2, respectively. To accurately identify the location of all barcodes and the Tn5 index, use the linker sequence between BC #2 and BC #3 as an anchor, allowing up to three mismatches.

- 35 Use a total of 96 cell barcodes. Since the last five bases of each barcode provide sufficient distinction, leverage these bases to enhance the demultiplexing rate. For each index, the corresponding Read 1 and Read 2 files were merged into a single FASTQ file to facilitate downstream analysis. Specifically, all reads associated with the same Tn5 index were concatenated into one FASTQ file, with both Read 1 and Read 2 sequences included. To retain cell-level information, the corresponding cellular barcode sequence was appended to the header line of each read. This ensured that both read sequences from the same fragment were stored together in the same FASTQ file, while preserving the association with their respective barcodes.

Pre-processing of demultiplexed single cell data

- 36 Implement the data processing workflow using Snakemake (**Ref 4**). Align sequencing reads to the reference genome (hg38 or mm10) using BWA (v0.7.17) (**Ref 5**) with the 'mem -k17' algorithm. Convert the resulting SAM files to BAM format, sort, and index using 'samtools (v1.17) (**Ref 6**) view', 'samtools (v1.17) sort', and 'samtools (v1.17) index'. Remove duplicates originating from both PCR and linear amplification using a custom in-house script. Remove low quality reads with 'samtools (v1.17) view -q 2'. Generate signal tracks in BigWig format with 'bamCoverage (v3.5.1)'. Perform transcription start site (TSS) enrichment analysis using 'computeMatrix (v3.5.1)', followed by heatmap visualization with 'plotHeatmap (v3.5.1)'. Convert BAM files to fragment files using an in-house script. Conduct peak calling using 'MACS2 (v2.1.2) callpeak' (**Ref 7**) with a q-value threshold of 0.05, and filter blacklist regions using 'bedtools (v2.30.0) intersect' (**Ref 8**). Calculate the fraction of reads within the TSS region (FRiT) using a window spanning -1000 bp to +200 bp relative to the TSS. For mouse samples, define high-quality cells as those with FRiT > 15 and >1500 unique fragments; for human samples, define high-quality cells as those with FRiT > 7 and >1000 unique fragments.

Single-Cell chromatin accessibility clustering and gene activity analysis

- 37 Perform single-cell clustering analysis using the SnapATAC2 (**Ref 9**) and Scanpy (**Ref 10**) packages. For mouse samples, import data using 'snap.pp.import_data' with the mm10 reference genome and filter out cells with TSS enrichment scores below 4. Use a 5 kb bin size to generate the tile matrix with 'snap.pp.add_tile_matrix'. For human samples, use the hg38 reference genome and a 10 kb bin size to construct the tile matrix.
- 38 To assess and remove potential doublets, select the top 250,000 features and estimate the doublet rate using 'snap.pp.scrublet', followed by filtering with 'snap.pp.filter_doublets'. Perform dimensionality reduction using spectral embedding ('snap.tl.spectral'), and conduct cell clustering with k-nearest neighbor (KNN) graph

construction 'snap.pp.knn', Leiden clustering 'snap.tl.leiden', and UMAP visualization 'snap.pl.umap'.

- 39 Calculate gene activity scores using 'snap.pp.makegenematrix' with upstream and downstream windows of 5000 bp and 500 bp, respectively. To annotate cell types, project cell type-specific marker gene activity onto the UMAP. Filter lowly expressed genes using 'sc.pp.filtergenes' with 'mincells=5', and perform gene activity normalization using 'sc.pp.normalize_total' followed by log transformation 'sc.pp.log1p'. Conduct data imputation and smoothing with the MAGIC algorithm 'sc.external.pp.magic'. Identify highly variable genes using 'scanpy.pp.highlyvariablegenes' with 'minmean=0.0125, maxmean=3, mindisp=0.5'. Determine differentially expressed genes using 'sc.tl.rankgenesgroups' with the t-test method, and use those with an adjusted p-value < 0.05 and log fold change (LogFC) > 0.25 to compute average expression per cell type and visualize as a heatmap.

Cell-type specific peaks identification

- 40 Perform peak calling using 'snap.tl.macs3' with 'groupby=CellType', and merge peaks using 'snap.tl.mergepeaks' with 'halfwidth=500'. Generate a peak-to-cell matrix using 'snap.pp.makepeakmatrix'. Normalize by sequencing depth for each cell. Cell type-specific peaks were identified using a one-versus-rest strategy, where peaks for each cell type (e.g., T cells) were compared against all remaining cells. Pseudo-bulk were constructed by randomly sampling 500 cells per condition and computing the average accessibility signal. Multiple testing correction was applied using the Benjamini-Hochberg method (multipletests with `fdr_bh`), and peaks were considered differentially accessible if they met both an FDR < 0.05 and a fold change > 2. Peaks showing significant differences across more than one cell type were excluded to ensure specificity.

Transcription factor enrichment for differential peaks

- 41 The peak-to-cell type matrix was used as input for chromVAR1(Ref 11). GC bias was corrected using 'addGCBias', and known motifs were retrieved from the JASPAR database using 'getJasparMotifs'. Background peaks were estimated with 'getBackgroundPeak', followed by deviation score computation using 'computeDeviations'. TF variability was assessed with 'computeVariability', and the top 100 most variable TFs were selected for visualization.

Protocol references

1. S. Picelli *et al.*, Tn5 transposase and tagmentation procedures for massively scaled sequencing projects. *Genome Res* **24**, 2033-2040 (2014).
2. Buenrostro, J.D., Giresi, P.G., Zaba, L.C., Chang, H.Y. & Greenleaf, W.J. Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. *Nat Methods* **10**, 1213-1218 (2013).
3. Ma, S. *et al.* Chromatin Potential Identified by Shared Single-Cell Profiling of RNA and Chromatin. *Cell***183**, 1103-1116 e1120 (2020).
4. J. Koster, S. Rahmann, Snakemake-a scalable bioinformatics workflow engine. *Bioinformatics* **34**, 3600 (2018).
5. H. Li, R. Durbin, Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* **25**, 1754-1760 (2009).
6. H. Li *et al.*, The Sequence Alignment/Map format and SAMtools. *Bioinformatics* **25**, 2078-2079 (2009).
7. Y. Zhang *et al.*, Model-based Analysis of ChIP-Seq (MACS). *Genome Biol* **9**, (2008).
8. A. R. Quinlan, I. M. Hall, BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics* **26**, 841-842 (2010).
9. K. Zhang, N. R. Zemke, E. J. Armand, B. Ren, SnapATAC2: a fast, scalable and versatile tool for analysis of single-cell omics data. *bioRxiv*, (2023).
10. F. A. Wolf, P. Angerer, F. J. Theis, SCANPY: large-scale single-cell gene expression data analysis. *Genome Biol* **19**, 15 (2018).
11. Schep, A.N., Wu, B., Buenrostro, J.D. & Greenleaf, W.J. chromVAR: inferring transcription-factor-associated accessibility from single-cell epigenomic data. *Nat Methods***14**, 975-978 (2017).