MuMu: a sample multiplexing protocol for droplet-based simultaneous single nuclei RNA- and ATAC-seq systems

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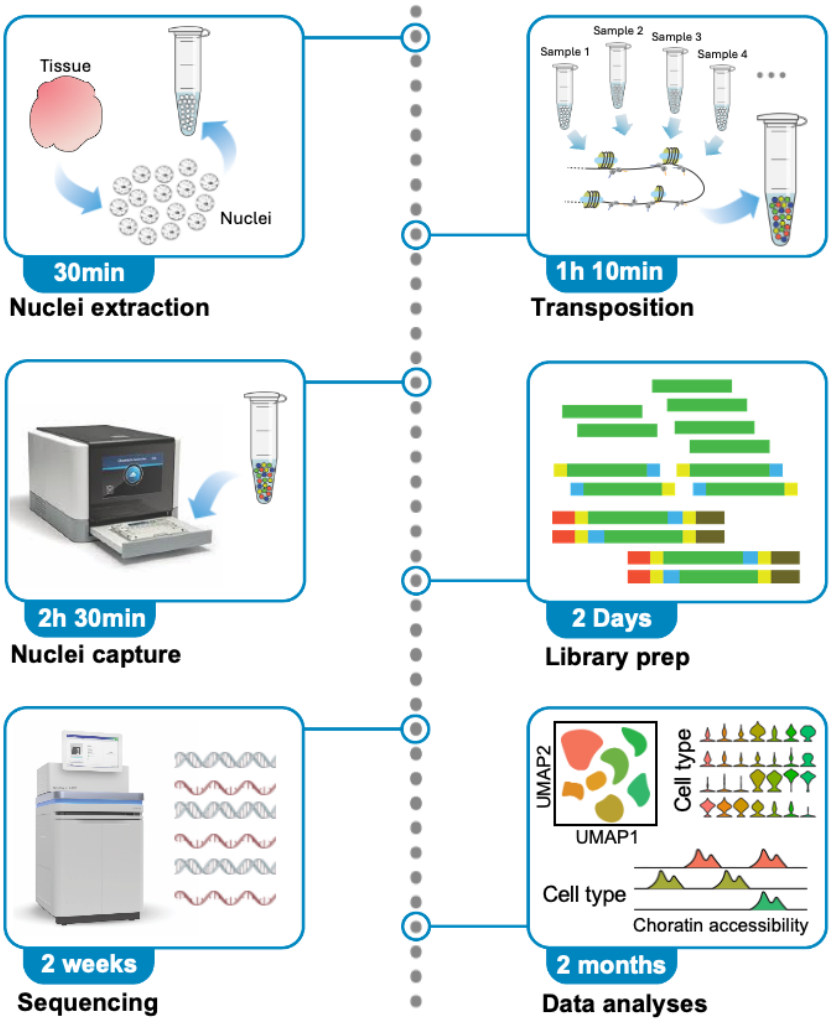
Summary
Sample multiplexing is a common approach to reduce experimental cost and technical batch effect. Here, we present a protocol that for the first time allows the pooling of single nuclei from multiple biological samples prior to performing simultaneous single nuclei RNA-seq and ATAC-seq, which we term *Mu*ltiplexed *Mu*ltiome (MuMu). We describe steps for assembling the custom Tn5 transposome, performing the transposition reaction, nuclei pooling, sequencing library preparation, and sequencing data pre-processing. This protocol will greatly reduce the cost of sn-Multiome.

Graphical abstract



Before you begin

The protocol below describes the specific steps for sample multiplexing before performing a sn-Multiome run with 10X Genomics Chromium Single Cell Gene Expression + ATAC kit. Sample multiplexing in this protocol refers to the action of combining single nuclei from multiple independent biological sources before performing droplet-based single nuclei capture and sequencing library preparation in a single reaction as if all the nuclei come from the same sample, significantly reducing the cost of analyzing multiple independent samples. Single cell multi-omics technologies have rapidly advanced in recent years.[1] Simultaneous single nuclei RNA and ATAC sequencing technology (referred to hereafter as multiome sequencing technology), in particular, is well established and commercially available.[3] However, the cost to perform one sn-Multiome reaction is very high. Moreover, biological experiments usually require multiple samples from different time points or conditions, in multiple replications. As a result, the cost to complete a whole experiment quickly rises to a prohibitive amount for many laboratories. Furthermore, when only a small

number of nuclei is available as input, the potential output of a sn-Multiome reaction is often under-utilized, exacerbating cost-inefficiency. With the release of high through-put single cell capture platforms such as the Chromium X series (10X Genomics), whose capture capacity goes up to millions of cells or nuclei per capture, a reliable sample multiplexing strategy is now even more relevant and valuable. Thus, we attempt to improve the utility of droplet-based sn-Multiome by redesigning the Tn5 transposon sequence to allow the insertion of a sample specific barcode during the initial transposition reaction of the ATAC workflow and prior to single nuclei capture. Later on, the sample-specific barcode can be reliably detected in the fastq files containing sequencing reads and easily incorporated into read alignment steps for demultiplexing purposes. While the current protocol is written based on and specifically for the 10X Genomics Chromium Single Cell ATAC + GEX Multiome kit, it can be easily adapted to other droplet-based sn-Multiome strategies, such as single nucleus ATAC-seq and CUT&TAG with minor modifications.[2] The same approach can also be used to multiplex bulk ATAC-seq samples.

Before you start, it is advisable that:

1. Institutional permission and oversight information for the animal study should be obtained. To demonstrate the outcome of the protocol, we collected samples from mouse neocortices at embryonic day (E) 16 and from pig neocortex at gestational week (GW) 10. The use of the animals was approved by the Institutional Animal Care and Use Committee (IACUC) at Children's National Research Institute.

2. In most cases, the protocol begins with a piece of frozen tissue or cell suspension. We typically collect tissues in cryopreservation vials (2ml), snap-frozen in liquid nitrogen and kept in -80°C freezer, which we assume as the starting point of the protocol. This allows for the independent storage of multiple samples that can be combined at the start of the Multiome workflow. Other methods of sample storage are applicable and should minimally affect the outcome of the procedure, as long as the integrity of genomic DNA and messenger RNA (mRNA) is ensured.

3. The current protocol requires the in-house assembly of transposome, which starts with naked Tn5 transposases. We recommend purchasing the enzyme from a reliable commercial source, but experimenters may also opt to produce homemade Tn5 transposase, which has the benefit of reducing overall experimental cost even further. In either case, the activity and purity of transposase should be confirmed before proceeding.

4. The most significant change we have made to a standard sn-Multiome workflow is the replacement of transposon with MuMu barcode oligomer. The key resource table contains four examples of MuMu barcode oligomers but an infinite numbers of such oligomers can be designed to meet the multiplexing need of specific experiments. The structure of the 42bp oligomers is: GTGCTCTTCCGATCTXXXXXXXXAGATGTGTATAAGAGACAG. Substitute "XXXXXXXX" with a barcode of choice. The length of the barcode does not need to be 8-bp. However, we noticed that when a transposon is 60 bp or longer, it forms concatenation during transposition reaction (Figure 1). The concatenated transposon repeats interfere with sequencing library preparation and cannot be removed simply by size selection without losing genomic fragments all together.

Transposon preparation

Timing: 30min

1. Mix 5μL ME-A (100μM) with 5μL ME-Rev (100μM) and 40μL TE buffer in a PCR tube.
2. Mix 5μL of each MuMu barcoding oligomer (100μM) with 5μL ME-Rev (100μM) and 40μL TE buffer in PCR strips or plates.
   Note: The number of MuMu barcoding oligomers depends on the number of samples to be multiplexed. Prepare at least one barcoding oligomer for one sample. More than one barcoding oligomer can be used for one sample.
3. Put all solutions on thermocycler and run the following program:

Step	Temperature	Time
Denaturation	98°C	00:03:00
Annealing	70°C	00:03:00
		Cooldown by 0.1□°C/sec
Hold	25°C	forever

Transposome assembly

Timing: 35min

4. Combine the following reagents in a PCR tube and mix by pipetting:

Reagent	Volume (μL)
Assembled ME-A/ME-Rev Transposon	1

Assembled MuMu-seq barcoding oligomer/ME-Rev Transposon	1
Tn5 transposase	10

Note: The volumes used in this table are typically enough for four reactions, assuming around 10,000 input nuclei per reaction.

5. Incubate at 25°C in a thermocycler for 30min.
   Note: The assembled transposome can be kept at -20°C for at least four weeks.

Prepare Pre-Amplification primers

Timing: 5min

6. Resuspend Pre-Amplification PCR primers to 100 µM.
7. Mix together 10µL of each primer and 60µL of TE buffer (100µL total).

Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Tn5	Varizyme	5000S https://www.varizymes.com/products/tn5-transposase?variant=43470943224056
Trypan Blue Solution, 0.4%	Gibco	15250061
TE buffer	Thermo Fisher	12090015
Q5® High-Fidelity 2X Master Mix	NEB	E5520S
NEBNext® Multiplex Oligos for Illumina® (Index Primers Set 1)	NEB	E7335S https://www.neb.com/en-us/products/e7335-nebnext-multiplex-oligos-for-illumina-index-primers-set-1
Nuclease-free H2O	Thermo Fisher	AM9916
1X PBS buffer solution	Thermo Fisher	10010023
Bovine serum albumin	Millipore-Sigma	A9418-5G
IGEPAL CA-630 detergent[a]	Abcam	ab285400
Tween-20 detergent[b]	Millipore-Sigma	11332465001
Digitonin detergent[c]	Promega	G9441
Protector RNase Inhibitor	Millipore-Sigma	3335399001
Critical commercial assays		
High Sensitivity D5000 ScreenTape	Agilent	5067-5592
High Sensitivity D5000 Reagents	Agilent	5067-5593

Chromium Next GEM Single Cell Multiome ATAC + Gene Expression	10XGenomics	
Experimental models: Organisms/strains		
Mouse: CD1	Charles River Laboratories	RRID: IMSR_CRL:022
Gottingen minipig		
Oligonucleotides		
DNA sequence: MuMu Barcode 1: GTGCTCTTCCGATCTAAACGGCGAGATGTGTATAAGAGACAG	This paper	N/A
DNA sequence: MuMu Barcode 2: GTGCTCTTCCGATCTCCTACCATAGATGTGTATAAGAGACAG	This paper	N/A
DNA sequence: MuMu Barcode 3: GTGCTCTTCCGATCTGGCGTTTCAGATGTGTATAAGAGACAG	This paper	N/A
DNA sequence: MuMu Barcode 4: GTGCTCTTCCGATCTTTGTAAGAAGATGTGTATAAGAGACAG	This paper	N/A
DNA sequence: ME_A: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG	This paper	N/A
DNA sequence: ME_rev: /5Phos/CTGTCTCTTATACACATCT	This paper	N/A
Primers for ATAC sequencing library amplification: Forward Primer: AATGATACGGCGACCACGAGATCTACAC	This paper	N/A
Primers for pre-amplification, see Table S1	This paper	N/A
Software and algorithms		
Cutadapt (v4.9 or newer)	https://cutadapt.readthedocs.io/en/stable/	N/A
Fastq-pair	https://github.com/linsalrob/fastq-pair/	N/A
Cellranger-arc	https://www.10xgenomics.com/support/software/cell-ranger-arc/latest	N/A
Seurat	https://satijalab.org/seurat/	N/A
Signac	https://stuartlab.org/signac/	N/A
Other		
5mL Polystyrene Round-Bottom Tube with Cell-Strainer (35µm) Cap	Fisher Scientific	352235
Bright-Line™ Hemacytometer	Millipore-Sigma	Z359629
Hand Held Tally Counter	Uline	H-7350
Tabletop Centrifuge	Eppendorf	5427R
4150 TapeStation System	Agilent	G2992AA; RRID:SCR_019393
Thermocycler	Eppendorf	2231000813
Optical tube strips (8x Strip)	Agilent	401428
Optical tube strips caps (8x Strip)	Agilent	401425

1.5-mL Microcentrifuge tubes	Eppendorf	022431021
Pipet-Lite LTS Pipette, L-2XLS+, L-20XLS+, L-200XLS+	Rainin	17014393, 17014392, 17014391

- ***a Supplied as a 10% solution***
- ***b Supplied as a 10% solution; store at 4C***
- ***c Supplied as a 2% solution in DMSO; (store at -20C)***

Materials and equipment setup

Lysis buffer

Reagent	**Final concentration**	**Amount**
1M Tris-HCL pH 7.4	10 mM	500 µL
5M NaCl	10 mM	100 µL
1M $MgCl_2$	3 mM	150 µL
10% IGEPAL	0.1%	500 µL
10% Tween-20	0.1%	500 µL
2% Digitonin	0.01%	250 µL
Protector RNase Inhibitor	0.2 U/µL	250 µL
1X PBS buffer solution	n/a	47.75 mL
Total	**n/a**	**50 mL**

Alternatives: Use reagents provided by 10X Genomics Chromium Nuclei Isolation Kit.

Nuclei Resuspension Buffer

Reagent	**Final concentration**	**Amount**
Bovine serum albumin powder	1% (wt/vol)	0.5g
0.5M Ethylenediaminetetraacetic acid (EDTA)	1mM	100 µL
Protector RNase Inhibitor	0.2 U/µL	250 µL
1X PBS buffer solution	n/a	Up to 50 mL
Total	**n/a**	**50 mL**

Alternatives: Use Nuclei Resuspension Buffer provided in the 10X Genomics Chromium Next GEM Single Cell ATAC + Gene Expression Multiome kit.

Step-by-step method details

Extract nuclei from each biological sample

Timing: 30min

This step extracts and isolates nuclei from frozen samples. The concentration of the final nuclei suspension is adjusted to a level that maximizes capture efficiency while minimizing the formation of doublets (capturing more than one nucleus per droplet).

1. Retrieve tissue or cells in a cryopreservation vial from -80°C freezer and immediately add 500µl of lysis buffer to the vial.
2. Incubate for 10min on ice.
3. After incubation, add 500µl nuclei resuspension buffer (RSB) and gently pipette 5-10 times until no visible cell lumps.
4. Adjust volume to 5ml and centrifuge at 800g for 5min to collect the nuclei.
5. Resuspend nuclei in RSB at appropriate volume (usually 1-2mL).
6. Filter resuspended nuclei into 5mL Polystyrene Round-Bottom Tube with Cell-Strainer Cap
7. Count nuclei with a hemacytometer.
    a. Mix 5µL nuclei suspension with 5µL Trypan Blue Solution 0.4% by gently pipetting at least 10 times.
    b. Load the mixture into the chamber on one side of the hemacytometer.
    c. Count the number of nuclei under a microscope and calculate the concentration.
8. Adjust nuclei concentration to about 1,000 nuclei/µL.

Transposing genomic DNA in the extracted nuclei with MuMu barcode

Timing: 1h 10min

This step transposes the open chromatin regions of genome within each nucleus and simultaneously adds the sample specific barcode. At the end of this step, the nuclei from multiple barcoded samples are combined and processed in a single reaction. As an example, we assume an experiment with four biological samples.

9. For each sample, combine the following reagents in a 1.5 mL microcentrifuge tube separately:

Reagent	Amount
Nuclei suspension	5 µL
ATAC Buffer B	7 µL
Tn5 transposase	1 µL
H_2O	2 µL

Note: If multiplexing more than four samples, adjust the number of input nuclei from each sample so that the final combined number does not exceed the maximum input amount required by the specific platform.

10. Incubate mixtures at 37°C for 1h on a thermocycler.
11. During incubation, prepare Master Mix.
    a. Combine reagents according to 10X Genomics Chromium Next GEM Single Cell Multiome ATAC + Gene Expression User Guide, Section 2.1a.
    b. Add 10 µL RSB to Master Mix and keep mixture on ice

    Note: If using 10X Genomics Chromium Next GEM Single Cell Multiome ATAC + Gene Expression kit, bring out Gel Bead from -80 °C freezer to equilibrate to room temperature.
12. Collect post-transposition nuclei by centrifugation.
    a. After the reaction, the nuclei in each tube are pelleted by centrifugation at 800g for 5 min.

    Critical: Record the tubes' orientation in the centrifuge. A pellet may not be visible but nuclei will be collected on the outside wall of the tube after centrifugation.

    b. Using a P20 pipette, remove all supernatant by pipetting from the opposite side of the pellet.
    c. Gently wash the outside wall of each centrifuge tube using the master mix prepared in step 3 and combine sequentially.
    d. Keep master mix containing all the transposed nuclei on ice.

Capture

Timing: 2h 30min

This step encapsules single nuclei within the droplet emulsions. Pre-mRNA and transposed genomic DNA fragments from each nucleus are released by lysis and captured on a gel bead. During reverse transcription, cell barcodes and unique molecular identifier (UMI) are added to mRNA (cDNA) and to genomic DNA fragments. Genomic DNA fragments and cDNA within the same droplet share the same cell barcode. Finally, the emulsion is broken and all molecules are collected for amplification in the next step.

13. The single cell capture is conducted faithfully following 10X Genomics Chromium Next GEM Single Cell Multiome ATAC + Gene Expression User Guide, Section 2.2 to Section 3.2.

Pause point: The product can be stored at 4 °C over night before proceeding to the next step.

Pre-amplification

Timing: 35min
This step amplifies the genomic DNA fragments and cDNA from the previous step. Specifically for genomic DNA fragments, additional sequences are added at the end as primer binding sites for sequencing library amplification.

14. Perform PCR reaction as follows:

PCR reaction master mix

Reagent	Amount
Product from Step 14	46 µL
Pre-amplification primers	4 µL
Q5® High-Fidelity 2X Master Mix	50 µL

Note: Do not use any "hot start" polymerase since the PCR reaction begins with an initial extension step.

PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Extension	72 °C	5 min	1
Initial Denaturation	98 °C	3 min	1
Denaturation	98 °C	20 sec	7 cycles
Annealing	58 °C	30 sec	
Extension	72 °C	1 min	
Final extension	72 °C	1 min	1
Hold	12 °C	forever	

15. Post pre-amplification cleanup: Follow 10X Genomics Chromium Next GEM Single Cell Multiome ATAC + Gene Expression User Guide, Section 4.3 to perform cleanup.

Pause point: Store at 4°C for up to 72 h or at −20°C for long-term storage.

ATAC Sequencing Library Amplification

Timing: 20 min

This step amplifies the genomic DNA fragments and adds sequencing adaptors at the end.

16. Perform PCR reaction as follows:

PCR reaction master mix

Reagent	Amount

Product from Step 15	40 µL
NEB Index	5 µL
ATAC Forward Primer (10 µM)	5 µL
Q5® High-Fidelity 2X Master Mix	50 µL

PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	98 °C	30 sec	1
Denaturation	98 °C	10 sec	7 cycles
Annealing	70 °C	20 sec	
Extension	72 °C	30 sec	
Final extension	72 °C	1 min	
Hold	12 °C	forever	1

ATAC Sequencing Library Post-amplification Cleanup

Timing: 20 min

In this step, SPRISelect beads are used to purify fragments with a double-sided size selection strategy. The final library consists of fragments mostly from open chromatin regions of the genome, with a minor population of single and multi-nucleosome fragments.

17. Cleanup amplified ATAC sequencing library with SPRIselect beads.
    a. Vortex to resuspend SPRIselect reagent. Add 60 µL SPRIselect reagent (0.6X) to each sample. Pipette mix.
    b. Incubate 5 min at room temperature.
    c. Place on the magnet until the solution clears.
    d. Transfer 150 µL supernatant to a new strip tube. DO NOT discard the supernatant.
    e. Vortex to resuspend SPRIselect reagent.
    f. Add 65 µL SPRIselect reagent (1.25X) to each sample (supernatant). Pipette mix.
    g. Incubate 5 min at room temperature.
    h. Place on the magnet until the solution clears.
    i. Remove the supernatant.
    j. Add 300 µL 80% ethanol to the pellet. Wait 30 sec.
    k. Remove the ethanol.
    l. Add 200 µL 80% ethanol to the pellet. Wait 30 sec.
    m. Remove the ethanol.
    n. Centrifuge briefly. Place on the magnet.
    o. Remove remaining ethanol.

p. Remove from the magnet. Immediately add 20.5 µl Buffer EB. Pipette mix.
q. Incubate 2 min at room temperature.
r. Centrifuge briefly. Place on the magnet until the solution clears.
s. Transfer 20 µL sample to a new tube strip.

Pause point: Store at 4°C for up to 72 h or at −20°C for long-term storage.

Gene Expression Sequencing Library Preparation

Timing: 2 days

This step amplifies the cDNA library, which is subsequently fragmented. Then, the cDNA fragments are ligated with sequencing adaptors and amplified again through PCR reaction to generate the final sequencing library.

18. Follow 10X Genomics Chromium Next GEM Single Cell Multiome ATAC + Gene Expression User Guide, Section 6 and 7 to generate gene expression sequencing library.

Sequencing Library Quality Control

Timing: 30 min

This is a routine quality control step before libraries are sequenced. Successful ATAC and gene expression sequencing libraries show typical size profiles of their corresponding library type. Refer to 10X Genomics Chromium Next GEM Single Cell Multiome ATAC + Gene Expression User Guide, Appendix: Agilent TapeStation Traces section for more details.

19. We recommend using an Agilent TapeStation with the High Sensitivity D5000 kits to perform quality control on the final ATAC and gene expression sequencing libraries. Follow the manufacturer's instructions depending on the exact instrument and kit used (TapeStation online protocol).

Sequencing

Timing: 2-4 weeks

20. We recommend sequencing both gene expression and ATAC libraries with a paired-end, dual index protocol.

Read 1 (R1)	150 cycles

i7 Index	6-8 cycles
i5 Index (R2)	24 cycles
Read 2 (R3)	150 cycles

Note: The i7 index corresponds to the NEB index and is only relevant during the fastq file generation step. The NEB index is 6-bp in length. If 8-bp is required, append “AT” at the end.

21. Provide the NEB index sequence from step 16 to generate fastq files with bcl2fastq function.
22. Three fastq.gz files will be generated after sequencing. The file names should be adjusted according to Cell Ranger ARC requirement as follows(‘X’ indicates a single digit):
    Read 1: [Run name]_SX_LXXX_R1_XXX.fastq.gz
    I5 index: [Run name]_SX_LXXX_R2_XXX.fastq.gz
    Read 2: [Run name]_SX_LXXX_R3_XXX.fastq.gz

Pre-process MuMu barcode sequences before read alignment

Timing: 2h

In this step, the fastq files are processed to prepare for alignment with the cellranger-arc function.

23. (Optional) Remove sequencing adaptors from R1 and R3.

```
> cutadapt -a CTGTCTCTTATA -a AGATCGGAAGAGC -G
  ^N{8}AGATGTGTATAAGAGACAG -l 8 -L 150 --discard-
  untrimmed  -o [temporary]_[Run name]_SX_LXXX_I1_XXX.fastq.gz
  -p [temporary]_[Run name]_SX_LXXX_R3_XXX.fastq.gz [Run
  name]_SX_LXXX_R3_XXX.fastq.gz [Run
  name]_SX_LXXX_R3_XXX.fastq.gz
```

24. Split fastq file of R3 into two temporary files corresponding to [temporary]_SX_LXXX_I1_XXX.fastq.gz that contains MuMu barcode sequence (“I1”) and the other one contains ATAC sequence (“R3”).

```
> cutadapt -a -G ^N{8}AGATGTGTATAAGAGACAG -l 8 -L 150 --
  discard-untrimmed -o [temporary]_[Run
  name]_SX_LXXX_I1_XXX.fastq.gz -p [temporary]_[Run
  name]_SX_LXXX_R3_XXX.fastq.gz [Run
  name]_SX_LXXX_R3_XXX.fastq.gz [Run
  name]_SX_LXXX_R3_XXX.fastq.gz
```

Note: The R3 file ([Run name]_SX_LXXX_R3_XXX.fastq.gz) is used twice as input for cutadapt to simplify the read splitting process. Refer to cutadapt user guide for more information (REF).

25. Generate sample specific fasta files corresponding to each MuMu barcode:

```
> cutadapt -g ^file:MuMu_barcodes.fa -e 1 --action=none -o [Run
  name]_{name}_ SX_LXXX_I1_XXX.fastq.gz -p [Run
  name]_{name}_SX_LXXX_R3_XXX.fastq.gz [temporary]_[Run
  name]_SX_LXXX_I1_XXX.fastq.gz [temporary]_[Run
  name]_SX_LXXX_R3_XXX.fastq.gz
```

Note: {name} will be replaced by sample name supplied in the MuMu_barcodes.fa, which can be modified by the user.

26. Unzip [Run name]_SX_LXXX_R1_XXX.fastq.gz, [Run name]_SX_LXXX_R2_XXX.fastq.gz and [Run name]_{name}_ SX_LXXX_I1_XXX.fastq.gz split the fastq files by MuMu barcodes using

```
> fastq_pair [Run name]_{name}_ SX_LXXX_I1_XXX.fastq [Run
  name]_SX_LXXX_R1_XXX.fastq
> gzip -c [Run name]_SX_LXXX_R1_XXX.fastq.paird.fq > [Run
  name]_{name}_SX_LXXX_R1_XXX.fastq.gz
> fastq_pair [Run name]_{name}_ SX_LXXX_I1_XXX.fastq [Run
  name]_SX_LXXX_R2_XXX.fastq
> gzip -c [Run name]_SX_LXXX_R2_XXX.fastq.paird.fq > [Run
  name]_{name}_SX_LXXX_R2_XXX.fastq.gz
```

fastq_pair function.
Note: fastq_pair outputs four files. Only the .paird.fq file of R1 (or R2) is useful here and needs to be renamed according to cellranger-arc format requirement of input files.

Note: all inputs need to be unzipped fastq files.

27. Make sure you have four sets of ATAC fastq files for each of the MuMu barcoded samples:

    [Run name]_{name}_SX_LXXX_R1_XXX.fastq.gz
    [Run name]_{name}_SX_LXXX_R2_XXX.fastq.gz
    [Run name]_{name}_SX_LXXX_R3_XXX.fastq.gz
    [Run name]_{name}_SX_LXXX_I1_XXX.fastq.gz

28. Run “cellranger-arc count” program for each sample, using the sample specific ATAC fastq files and combined gene expression fastq files as input:

```
cellranger-arc count \
   --reference={genome_reference} \
   --id=[Run name]_{name} \
   --libraries=libraries.csv
```

29. (Optional) Run cellranger-arc aggr to aggregate all samples into one dataset.

Expected outcomes

The sn-Multiome ATAC library generated with the MuMu protocol is identical to ones that are generated with the standard 10X Genomics Chromium Next GEM Single Cell Multiome ATAC + Gene Expression workflow (Figure 2). Sometimes a smaller peak (less than 150 bp) appears in the final library, which represents adaptor dimer and should not affect sequencing or downstream analyses.

Quantification and statistical analysis

As a demonstration of the MuMu protocol, we processed tissue samples obtained from a mouse neocortex at E16 and a pig neocortex at gestation week (GW) 10. The nuclei from the two pieces of tissue samples were extracted. We then performed transposition reaction with the MuMu barcode oligomers as the transposon on two separate pools of nuclei from each tissue sample. The two mouse nuclei pools correspond to sample 1 and sample 3, while the pig nuclei pools correspond to sample 2 and sample 4, respectively. Following the MuMu preparation and bioinformatics preprocessing pipeline, more than 90% of all the raw ATAC reads contained correct MuMu barcodes and were confidently assigned to their corresponding sample (Figure 3A). We first assessed the rate at which MuMu barcodes mix with one another. When RNA or ATAC reads from any nucleus are mapped to the appropriate genome reference (mouse-to-mouse or pig-to-pig), the alignment rate is around 90%. However, when reads are mapped to the opposing reference, the alignment rate is only around 20%, due to heterology between mouse and pig genomes. We demonstrated this pattern by mapping a mouse sn-Multiome dataset (“10X Multiome”) generated in-house using the original 10X Genomics Multiome protocol to both the mouse and the pig genome references (Figure 3B). Based on this observation, we reasoned that if there is frequent mixing between MuMu barcodes, one would expect a substantial shift in the distribution of mapping ratio and that more nuclei would be assigned to one species but have their reads mapped to the genome of the opposing species. Our analysis indicated that all the nuclei displayed the correct pattern of mapping ratio based on the percentage of ATAC reads mapped to the two genomes, suggesting that there is no mixing or interchange of the MuMu barcodes after the transposition reactions. While RNA reads from a small number (4.3%) of the total nuclei mapped mainly to the opposing genome, none of these nuclei had a mapping rate of the ATAC reads to the opposing

genome at more than 50%, an average of 20.1% (SE±0.1%). The erroneously mapped RNA reads were most likely due to ambient RNA. This phenomenon can be used to exclude this minority of nuclei. Together, our data demonstrated that the MuMu barcodes faithfully and specifically labeled the samples they were assigned to. The nuclei with more than 50% of either RNA or ATAC reads mapped to the opposing genome were removed from downstream analyses.

A unique advantage of the MuMu barcoding system is its ability to unambiguously detect doublets, due to its reliability. Thus, we next examined how many cell barcodes (CB) were assigned to more than one sample as an indication of doublets. We found 144 CB shared between Sample 1 and Sample 3, as well as 571 CB shared between Sample 2 and Sample 4 (Figure 3C). The doublet rates were therefore about 5% between the mouse samples and about 20% between the pig samples. While the doublet ratio of the pig samples was higher than the mouse samples, it was still within the expected range of a droplet based single cell experiment[4], and may reflect an underestimation of the nuclei concentration during the extraction procedures for one of the pig samples (Sample 4) due to human error. We removed any CB that was assigned to more than one sample from downstream analyses.

The remaining nuclei displayed compatible distributions of RNA read counts, detected gene counts and percentage of RNA reads mapped to mitochondrial genome to standard single cell RNA-seq datasets. We then performed the Seurat (v5) analyses pipeline[5] and identified cell clusters in uniform manifold approximation and projection graphs (Figure 3E and F). The cell clusters from either species expressed the expected marker genes appropriate for the neocortical developmental period. Notably, E16 in mouse and GW10 in pig mark the beginning gliogenesis in the two species (Figure 3G and H). As expected, we identified oligodendrocyte precursor cell populations (Mm_2 and Ss_8) in both datasets, which were marked by a specific expression of *PTPRZ1*. The ATAC data also displayed compatible quality to standard single cell ATAC-seq datasets, reflected by the distributions of ATAC read count, number of ATAC features, transcription start site enrichment and nucleosome signal. We then examined the chromatin accessibility landscape around the gene body of *PTPRZ1*, we observed an enhanced openness of the *PTPRZ1* locus. More importantly, in both species, we were able to identify an ATAC peak about 200,000bp upstream of the gene that correlated with the *PTPRZ1* RNA expression (Figure 3J and K).[6] The analyses thus identify a potentially common regulatory element of *PTPRZ1* shared by the two species.

Limitations

The limitations commonly associated with snATAC-seq and snRNA-seq also apply to the protocol described here. We want to highlight one particular limitation that pertains to the transposition reaction. Current state-of-the-art ATAC-seq protocols, including the one described here, utilize transposon oligomers with two different sequences (mixed at equal molarity), so that DNA fragments can be tagged with different oligomers on each end to serve as primer binding sites for the PCR amplification. As a result, however, about half of the transposition events generate DNA fragments with

the same oligomer sequences at both ends, and these “homo-tagged” fragments cannot be amplified. Thus, 50% of the transposed DNA fragments are lost by default.[7] This is not a significant drawback in bulk-tissue ATAC-seq because there are presumably many copies of the genome from the large amount of nuclei with the same open chromatin configuration. However, at the single nucleus level, each genome is considered unique, which means the open chromatin fragments from each nucleus are also unique. The loss of 50% fragment from each single nucleus is irreversible and poses a challenge on the interpretation of final sequencing data.

A limitation specific to the current protocol is the randomness in the sequencing read distribution and nuclei distribution among the MuMu barcoded samples, which is affected by many factors. The sequencing and nuclei distributions are only accessible after sequencing, at which point it is already too late to make an adjustment. This limitation emphasizes the importance of accurate estimation of nuclei concentration during the extraction steps and precise techniques in pipetting nuclei between steps. The transposition reaction, especially the amount of transposome used, should also be carefully tested to achieve the most optimal number of fragments from open chromatin regions. It may be advisable to always perform a test sequencing run at low read depth to see the distributions before committing to a high throughput run.

Troubleshooting

Problem 1:

Failed to see a multimodal ATAC sequencing library profile. Only one peak can be detected at around 200 bp (Step 19).

This type of library profile usually indicates degradation of chromatin structure, resulting from poor quality of starting material. It is also possible that too much transposome is used. Another possible cause is an error during ATAC sequencing library post-amplification cleanup.

Potential solution:

- Collect fresh tissue. Be sure to snap-frozen sample as soon as possible in liquid nitrogen and transfer it to -80 °C right away after freezing for long term storage.
- Make sure to perform all nuclei extraction procedure on ice. Centrifuge nuclei at 4 °C until transposition step.
- When in doubt, perform a bulk ATAC-seq with sample in question to assess quality before proceeding to sn-Multiome.
- Try reducing the amount of transposome during the transposition step.
- During ATAC sequencing library post-amplification cleanup, make sure SPRIselect beads are well mixed every time and use the recommended ratio of beads to sample.

Problem 2:

Nuclei clump after extraction and cannot be dissociated further (Step 1-8).

This is usually caused by over-lysis resulting in the release of genomic DNA from broken nuclei, due to excessive pipetting or strong lysis buffer.

Potential solution:

- Pipette gently during nuclei extraction steps and reduce pipetting frequency depending on tissue type.
- Reduce the amount of detergent in the lysis buffer.
- Add DNase I to lysis buffer may help digest free-floating genomic DNA and reduce tangling.

Problem 3:

The final concentration of ATAC sequencing library is low (Step 14-16).

This usually indicates an overestimation of the number of captured nuclei.

Potential solution:

- Quantification of starting number of nuclei: The initial estimation of nuclei concentration needs to be accurate. Carefully count and calculate the concentration of nuclei after extraction.
- Sufficiently resuspend nuclei containing solution to homogeneity before mixing transposition reaction.
- Carefully collect nuclei after transposition with master mix.
- Further amplify sequencing library with Illumina P5 and P7 primers.

Resource availability

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Tarik F. Haydar, PhD, thaydar@bu.edu

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Zhen Li, PhD, zli5@bu.edu

Data and code availability

The datasets described in this protocol can be found on SRA with accession number PRJNA1191389.

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Author contributions

Z. L. designed and executed all experiments, including bioinformatics analyses. T.F.H. supervised the project. Z. L. and T. F. H. composed the manuscript.

Declaration of interests

The authors declare no competing interests.

References

1. Baysoy, A., Bai, Z., Satija, R., and Fan, R. (2023). The technological landscape and applications of single-cell multi-omics. Nature Reviews Molecular Cell Biology 2023 24:10 *24*, 695–713. https://doi.org/10.1038/s41580-023-00615-w.
2. Wu, S.J., Furlan, S.N., Mihalas, A.B., Kaya-Okur, H.S., Feroze, A.H., Emerson, S.N., Zheng, Y., Carson, K., Cimino, P.J., Keene, C.D., et al. (2021). Single-cell CUT&Tag analysis of chromatin modifications in differentiation and tumor progression. Nature Biotechnology 2021 39:7 *39*, 819–824. https://doi.org/10.1038/s41587-021-00865-z.
3. Single Cell Multiome ATAC + Gene Expression - Official 10x Genomics Support https://www.10xgenomics.com/support/single-cell-multiome-atac-plus-gene-expression.
4. Xi, N.M., and Li, J.J. (2021). Benchmarking Computational Doublet-Detection Methods for Single-Cell RNA Sequencing Data. Cell Syst *12*, 176-194.e6. https://doi.org/10.1016/J.CELS.2020.11.008.
5. Hao, Y., Stuart, T., Kowalski, M.H., Choudhary, S., Hoffman, P., Hartman, A., Srivastava, A., Molla, G., Madad, S., Fernandez-Granda, C., et al. (2023). Dictionary learning for integrative, multimodal and scalable single-cell analysis. Nature Biotechnology 2023 42:2 *42*, 293–304. https://doi.org/10.1038/s41587-023-01767-y.
6. Stuart, T., Srivastava, A., Madad, S., Lareau, C.A., and Satija, R. (2021). Single-cell chromatin state analysis with Signac. Nature Methods 2021 18:11 *18*, 1333–1341. https://doi.org/10.1038/s41592-021-01282-5.
7. Penkov, D., Zubkova, E., and Parfyonova, Y. (2023). Tn5 DNA Transposase in Multi-Omics Research. Methods Protoc *6*, 24. https://doi.org/10.3390/MPS6020024.

Figure legends

Figure 1. Tn5 transposome concatenate during reaction when transposon is 60 base pairs (bp) in size.

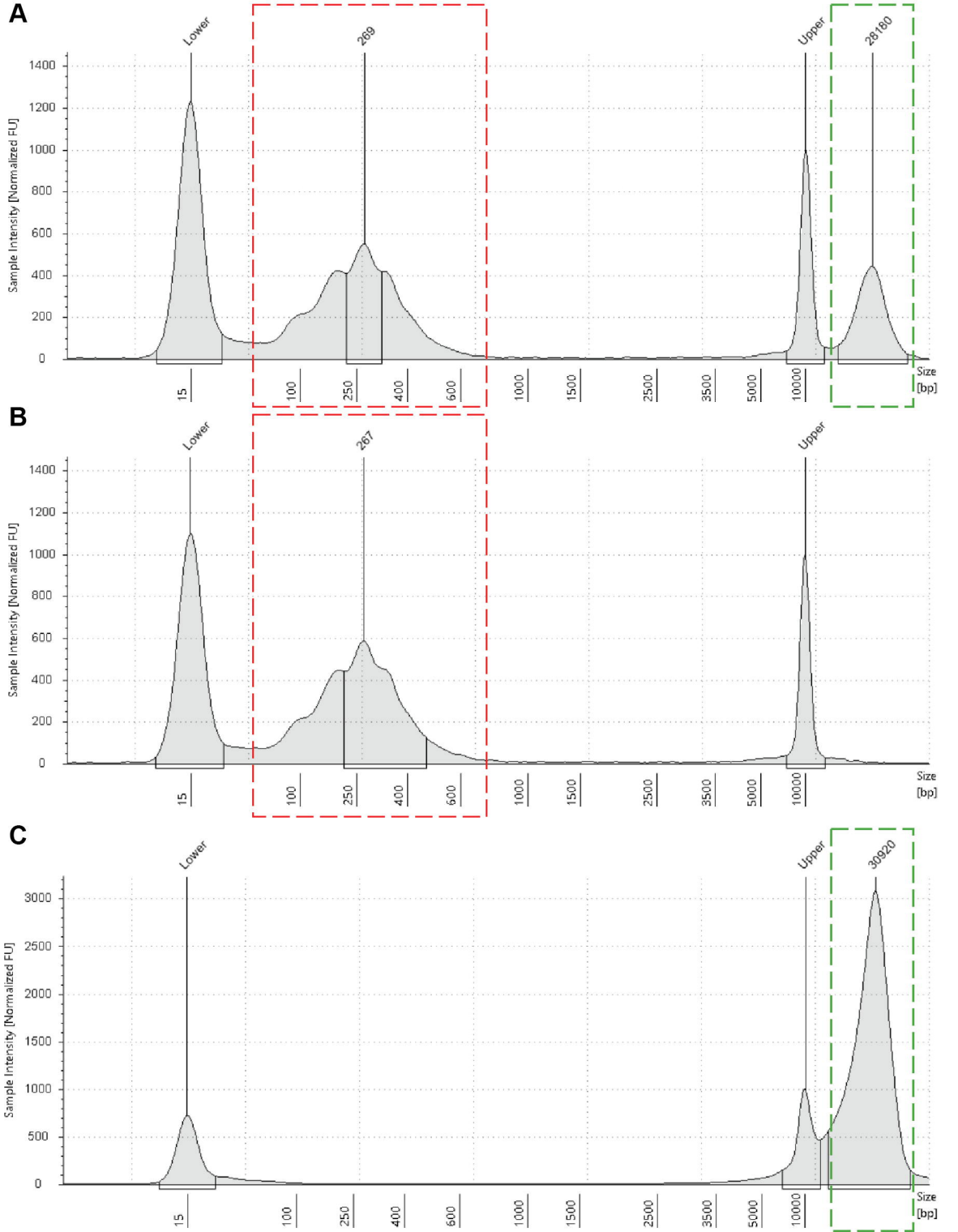
(A and B) TapeStation analysis traces showing unexpected products (rectangle with red dashed line) with periodicity of roughly 60bp in the presence (A) or absence (B) of genomic DNA (rectangle with green dashed line), which the transposon is at 60bp.

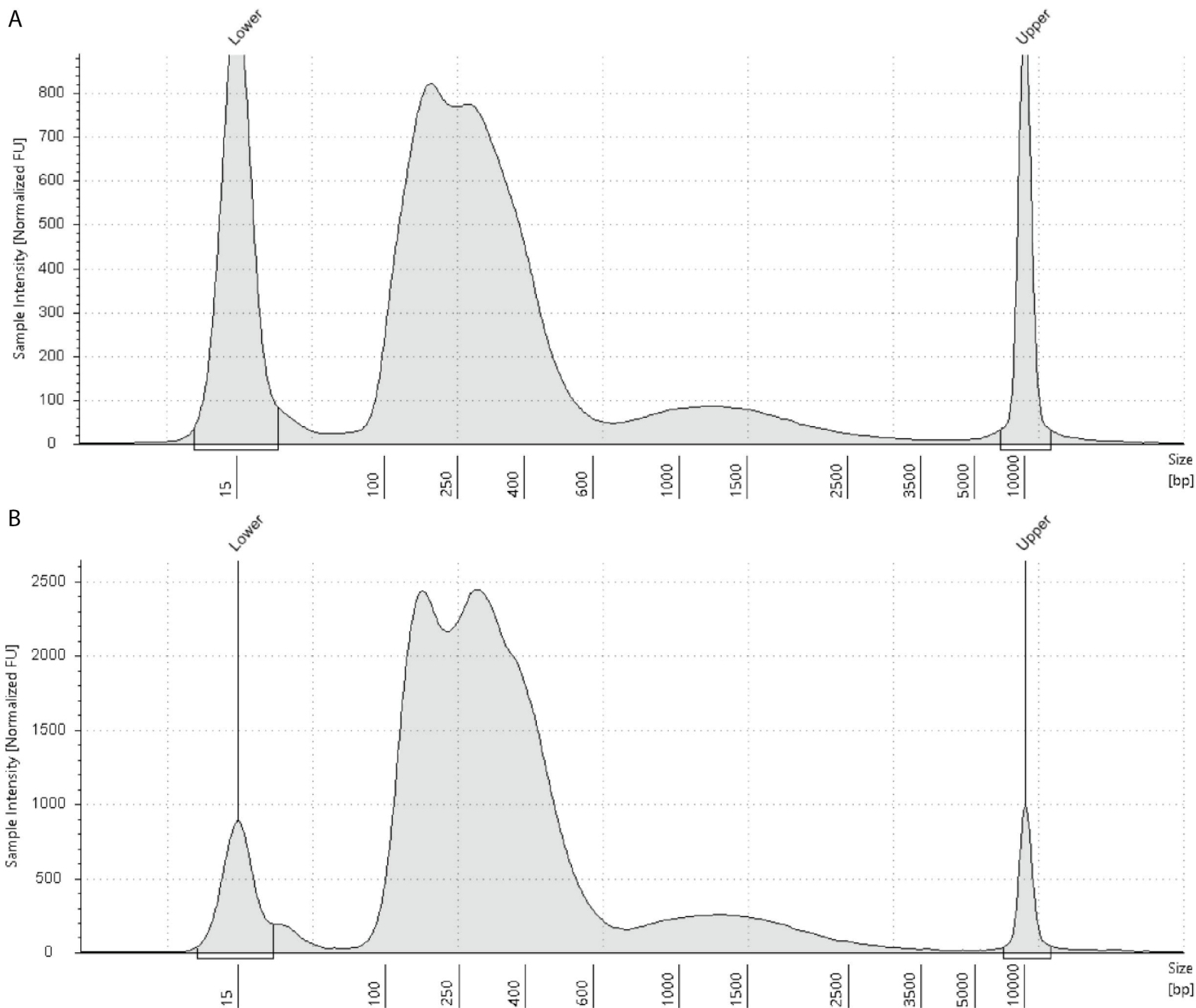
(C) No concatenation is observed using the MuMu barcode oligomers, which are 45 bp in size.

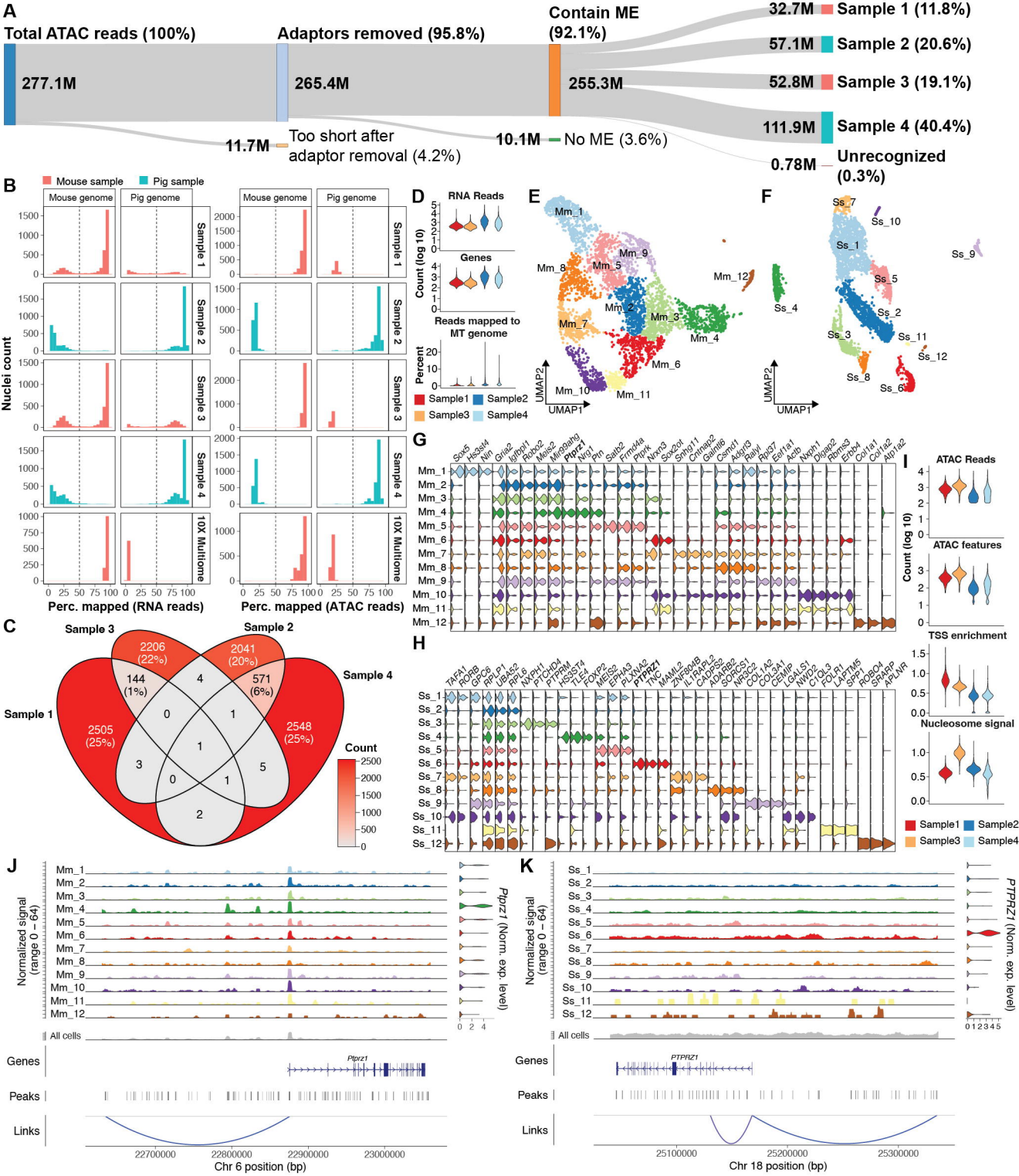
Figure 2. Typical TapeStation traces of finished ATAC libraries produced using (A) the current protocol or (B) the original 10X Genomics Chromium Next GEM Single Cell Multiome ATAC + Gene Expression protocol.

Figure 3. Demonstration of sample multiplexing and downstream data analyses.
(A) Sankey diagram showing the breakdown of all ATAC reads acquired through sequencing at each data preprocessing step. The percentage out of the total ATAC reads for each category is labeled in parentheses. ME, mosaic end; M, million reads.
(B) Histograms showing the distribution of percentages of RNA or ATAC reads mapped to mouse or pig genome from each nucleus. Sample 1 and 3 are collected from a mouse neocortex at embryonic day (E) 16, while 2 and 4 are from a pig neocortex at gestation week 10. Dotted vertical lines indicate 50% mapping rate ("cutoff"). Additionally, an E15.5 mouse neocortex sample ("10X Multiome") processed using the original 10X Genomics Chromium Next GEM Single Cell Multiome ATAC + Gene Expression kit was included for comparison.
(C) Venn diagram showing number of cell barcodes presented in each sample. Cell barcodes presented in more than one sample are likely "doublets" and are removed from downstream analyses.
(D) Violin plots showing distributions of RNA read counts, detected genes, and percentage of reads mapped to mitochondrial genome in nuclei passing quality control. MT, mitochondria.
(E and F) Uniform manifold approximation and projection constructed with gene expression data showing single nuclei profiles from the mouse (C) or the pig (D) samples. Colors represent cell clusters identified through the Seurat analysis pipeline.
(G and H) Violin plots showing the most specifically expressed genes associated with each cell cluster of the mouse (E) or the pig (F) samples. Clusters are arranged by size from largest to smallest.
(I) Violin plots showing distributions of ATAC read counts, ATAC features, transcription start site enrichment, and nucleosome signal. TSS, transcription start site.
(J and K) Chromatin accessibility profiles around gene *PTPRZ1* in each cell cluster of the mouse (J) or the pig (K) samples. *PTPRZ1* expression levels are shown on the right as violin plots. The accumulative chromatin accessibility profile from all nuclei is shown below in dark grey., followed by a diagram of *PTPRZ1* gene body. All identified ATAC peaks ("Peaks") within the region are shown as vertical lines. A peak in the upstream region ("Links") is identified in both mouse and pig genome that is correlated with gene expression of *PTPRZ1*. Norm. exp. level, normalized expression level.

Table 1: Pre-amplification primers


A
Lower
269
Upper
28180
Sample Intensity [Normalized FU]
1400
1200
1000
800
600
400
200
0
15
100
250
400
600
1000
1500
2500
3500
5000
10000
Size [bp]
B
Lower
267
Upper
Sample Intensity [Normalized FU]
1400
1200
1000
800
600
400
200
0
15
100
250
400
600
1000
1500
2500
3500
5000
10000
Size [bp]
C
Lower
Upper
30920
Sample Intensity [Normalized FU]
3000
2500
2000
1500
1000
500
0
15
100
250
400
600
1000
1500
2500
3500
5000
10000
Size [bp]


A
Lower
Upper
Sample Intensity [Normalized FU]
800
700
600
500
400
300
200
100
0
15
100
250
400
600
1000
1500
2500
3500
5000
10000
Size
[bp]
B
Lower
Upper
Sample Intensity [Normalized FU]
2500
2000
1500
1000
500
0
15
100
250
400
600
1000
1500
2500
3500
5000
10000
Size
[bp]


A
Total ATAC reads (100%)
277.1M
Adaptors removed (95.8%)
265.4M
11.7M
Too short after adaptor removal (4.2%)
Contain ME (92.1%)
255.3M
10.1M
No ME (3.6%)
32.7M
Sample 1 (11.8%)
57.1M
Sample 2 (20.6%)
52.8M
Sample 3 (19.1%)
111.9M
Sample 4 (40.4%)
0.78M
Unrecognized (0.3%)
B
Mouse sample
Pig sample
Mouse genome
Pig genome
Sample 1
Sample 2
Sample 3
Sample 4
10X Multiome
Nuclei count
Perc. mapped (RNA reads)
Perc. mapped (ATAC reads)
C
Sample 1
Sample 2
Sample 3
Sample 4
2505 (25%)
2206 (22%)
2041 (20%)
2548 (25%)
144 (1%)
571 (6%)
Count
D
RNA Reads
Genes
Reads mapped to MT genome
Count (log 10)
Percent
Sample1
Sample2
Sample3
Sample4
E
UMAP1
UMAP2
F
G
H
I
ATAC Reads
ATAC features
TSS enrichment
Nucleosome signal
J
Normalized signal (range 0 – 64)
Ptprz1 (Norm. exp. level)
All cells
Genes
Peaks
Links
Chr 6 position (bp)
K
PTPRZ1 (Norm. exp. level)
Chr 18 position (bp)

Oligonucleotides		
Primers for pre-amplification: cDNA Forward: CTACACGACGCTCTTCC	This paper	N/A
Primers for pre-amplification: cDNA Reverse: AAGCAGTGGTATCAACG	This paper	N/A
Primers for pre-amplification: ATAC Forward: AATGATACGGCGACCAC	This paper	N/A
Primers for pre-amplification: ATAC Reverse: CAGACGTGTGCTCTTCCGATCT	This paper	N/A