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Protocol extension

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Spatially resolved genome-wide joint profiling of epigenome and transcriptome with spatial-ATAC-RNA-seq and spatial-CUT&Tag-RNA-seq

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Abstract

The epigenome of a cell is tightly correlated with gene transcription, which controls cell identity and diverse biological activities. Recent advances in spatial technologies have improved our understanding of tissue heterogeneity by analyzing transcriptomics or epigenomics with spatial information preserved, but have been mainly restricted to one molecular layer at a time. Here we present procedures for two spatially resolved sequencing methods, spatial-ATAC-RNA-seq and spatial-CUT&Tag-RNA-seq, that co-profile transcriptome and epigenome genome wide. In both methods, transcriptomic readouts are generated through tissue fixation, permeabilization and in situ reverse transcription. In spatial-ATAC-RNA-seq, Tn5 transposase is used to probe accessible chromatin, and in spatial-CUT&Tag-RNA-seq, the tissue is incubated with primary antibodies that target histone modifications, followed by Protein A-fused Tn5-induced tagmentation. Both methods leverage a microfluidic device that delivers two sets of oligonucleotide barcodes to generate a two-dimensional mosaic of tissue pixels at near single-cell resolution. A spatial-ATAC-RNA-seq or spatial-CUT&Tag-RNA-seq library can be generated in 3–5 d, allowing researchers to simultaneously investigate the transcriptomic landscape and epigenomic landscape of an intact tissue section. This protocol is an extension of our previous spatially resolved epigenome sequencing protocol and provides opportunities in multimodal profiling.

This protocol is an extension to: *Nat. Protoc.* **19**, 3389–3425 (2024): https://doi.org/10.1038/s41596-024-01013-y

Key points

- Spatially resolved concurrent profiling of both transcriptome and epigenome is essential for studying spatio-temporal regulation of gene expression, but the technical solutions permitting such analyses remain limited.
- This protocol describes spatial-ATAC-RNA-seq and spatial-CUT&Tag-RNA-seq, which profile genome-wide transcription jointly with open chromatin and histone modifications, respectively, on a tissue section.

Key references

Zhang, D. et al. Spatial epigenome–transcriptome co-profiling of mammalian tissues. *Nature* **616**, 113–122 (2023): https://doi.org/10.1038/s41586-023-05795-1

Farzad, N. et al. Spatially resolved epigenome sequencing via Tn5 transposition and deterministic DNA barcoding in tissue. *Nat. Protoc.* **19**, 3389–3425 (2024): https://doi.org/10.1038/s41596-024-01013-y

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Introduction

In mammalian tissues, epigenetic regulation of gene expression governs diverse biological processes[1,2]. The relationship between gene expression and these epigenetic factors is controlled in a spatio-temporal manner and studying genome-wide transcriptomics–epigenomics dynamics is crucial for researchers to understand transcription initiation and cell state maintenance in health, disease and development. Recent advances in single-cell multimodal technologies, such as co-profiling of transcriptomics and chromatin accessibility with the droplet microfluidics or split-pool barcoding platforms[3–6], have expanded our knowledge of tissue heterogeneity, disease responses and cell fate decisions[7–9]. Nevertheless, spatial information is lost due to harsh tissue dissociation applied in these methods, which prevents our understanding of how gene transcription is epigenetically regulated within a complex tissue architecture.

Development of the protocol

Therefore, spatially resolved multiomics has begun to address this gap by measuring different modalities of '-omics' on an intact tissue section, with either sequencing- or imaging-based approaches[10–13]. Our group previously developed deterministic barcoding in tissue for spatial '-omics' sequencing (DBiT-seq) to profile the whole transcriptome and a panel of proteins simultaneously in situ[14,15]. DBiT-seq uses two microfluidic channel array chips that are perpendicular to each other, to divide a tissue section into a two-dimensional (2D) grid of spatially encoded oligonucleotide-barcoded pixels. The deterministic barcoding method has become an openly accessible platform and has been used reproduced or modified by other groups[16,17]. Based on this platform, we also developed methods to profile the epigenomic modality at the spatial level, including spatially resolved assay for transposase-accessible chromatin with sequencing (ATAC-seq)[18–20], a technology that captures open chromatin accessibility) and cleavage under targets and tagmentation (CUT&Tag)[21,22], a representative technology currently widely used for histone modification profiling) (that is, spatial-ATAC-seq and spatial-CUT&Tag[23–25]). However, both methods are limited to one epigenomic modality and lack the gene expression readout, which constrains the study of transcriptome–epigenome correlation and hampers characterization of cell identities.

Motivated by this, we recently developed methods for spatially resolved genome-wide transcriptomics jointly profiled with chromatin accessibility (spatial-ATAC-RNA-seq) or histone modifications (spatial-CUT&Tag-RNA-seq)[26], and used them to study embryonic and juvenile mouse brain and adult human brain tissues. Here, we present a step-by-step detailed protocol for spatial-ATAC-RNA-seq and spatial-CUT&Tag-RNA-seq to enable generation of spatial multiomics data with high quality.

Applications of the method

Both single-cell RNA sequencing (scRNA-seq) and scATAC-seq, as well as single-cell multiomic methods that co-map the two modalities, have been demonstrated in a variety of samples, including embryonic cells, tumors and various organs and tissues[5,27–30]. scCUT&Tag has been applied on mouse brain, human blood and embryonic stem cells and human brain tumors, and the bulk CUT&Tag assay is more comprehensively validated in various sample types[31–35]. We therefore envision that spatial-ATAC-RNA-seq and spatial-CUT&Tag-RNA-seq can be applied in diverse biomedical research settings for unbiased characterization of both transcriptomics and epigenomics to enable investigation of the spatio-temporal relationships between the two modalities.

In our recent work[26], we performed spatial-ATAC-RNA-seq and spatial-CUT&Tag-RNA-seq on fresh-frozen embryonic day 13 (E13) mouse embryos, mouse postnatal day 21/22 (P21/22) brain and adult human brain (hippocampus region) samples at a resolution of 20 μm or 50 μm pixel size, demonstrating that near single-cell resolution can be achieved with the 20 μm microfluidic channel array chip and that the 50 μm microfluidic chip can cover a larger area of region of interest (ROI). Antibodies against H3K27me3, H3K27ac or H3K4me3 histone modifications

were used in the spatial-CUT&Tag-RNA-seq experiment, targeting genomic regions that mostly represent repressed loci, active promoters or enhancers, and active promoters, respectively. We performed dimensional reduction and clustering analysis on the spatial-ATAC-RNA-seq and spatial-CUT&Tag-RNA-seq data[26]. Many genes show expression levels consistent with their chromatin accessibility or histone modification profiles, and we identified a high degree of concordance in spatial cell type distribution determined by each modality. This method can also be used to identify inconsistencies between transcriptomics and epigenomics at the spatial level. For example, our work demonstrated that some genes (for example, *Sox10*, *Cux2*, *Neurod6*) show low or undetectable expression even with high chromatin accessibility in the P22 mouse brain[26].

Compared with our recently described protocol for spatial-ATAC-seq and spatial-CUT&Tag[25], the integration of reverse transcription (RT), template switching and streptavidin-bead affinity pulldown procedures enables the simultaneous co-profiling of epigenomic and transcriptomic data, while preserving the quality of the epigenomic readouts. This advancement is critical, as multimodal co-profiling is essential for investigating gene expression regulatory mechanisms and constructing gene regulatory networks[36], and recent single-cell multiomics studies have suggested that epigenomic profiling alone is less effective for cell type classification without complementary transcriptomic data[37,38]. In datasets generated from transcriptomics-epigenomics co-profiling assays, RNA-seq data enhances accurate cell type annotation, facilitating the analysis of fragment distribution across cell types and differential peak identification in epigenomic analysis[39]. Therefore, the use of spatial-ATAC-RNA-seq or spatial-CUT&Tag-RNA-seq is recommended in the following scenarios: (1) when the goal is to explore associations between the two modalities in a spatio-temporal context; (2) when computational tissue co-registration is challenging, making separate spatial transcriptomics and epigenomics profiling less advisable; or (3) when sample material is limited, as the protocol presented allows the extraction of both transcriptomic and epigenomic data from a single sample. Conversely, the single-modality epigenomic sequencing protocol[25] may be preferable when high-quality spatial transcriptomics data (or scRNA-seq data) are available and can serve as a reference for multimodal integrative analysis and cell type annotation[40]. The single-modality approach offers benefits in reduced reagent costs and is less labor intensive than the procedures described in this Protocol Extension. Additionally, for samples with compromised RNA quality, such as those with low RNA integrity numbers, spatial-ATAC-seq or spatial-CUT&Tag remain feasible options, and we recommend this single-modality epigenomic profiling in such cases.

Comparison with other methods

Spatial-ATAC-RNA-seq and spatial-CUT&Tag-RNA-seq can detect 1,000–2,000 genes and 2,000–5,000 unique molecular identifiers (UMIs) per 20 µm pixel, comparable to many current scRNA-seq studies on primary tissues[41], and we demonstrated the feasibility of dimensional reduction and unsupervised single-cell level clustering analysis on these data. In spatial-ATAC-RNA-seq experiments, ~15,000 unique fragments were obtained per 20 µm pixel with a fraction of mitochondrial reads lower than 5% and nearly 20% of these unique fragments were enriched in the transcription start site (TSS) regions, consistent with our previous single-modality spatial-ATAC-seq technology[24].

In spatial-CUT&Tag-RNA-seq experiments, we obtained ~10,000 unique fragments per 20 µm pixel for H3K27ac and H3K27me3 modifications, which is comparable to our previous spatial-CUT&Tag technology[23]. On the basis of our recent work[26], H3K4me3 profiling in spatial-CUT&Tag-RNA-seq may present a lower number of unique fragments per pixel than that in spatial-CUT&Tag, although we observed a higher fraction of H3K4me3 fragments located in peaks, suggesting a higher specificity.

MISAR-seq (microfluidic indexing-based spatial assay for transposase-accessible chromatin and RNA-sequencing)[17] is another spatially resolved sequencing method for co-mapping of transcriptomics and chromatin accessibility that is based on a similar 50-channel × 50-channel deterministic barcoding microfluidic design. MISAR-seq was used to study developing mouse brains, demonstrating gene detection sensitivity and genomic coverage comparable to our spatial-ATAC-RNA-seq, although MISAR-seq has not been tested for either histone modification profiling or human sample applications and the resolution was limited at the 50 µm pixel size.

In addition, our spatial-ATAC-RNA-seq and spatial-CUT&Tag-RNA-seq protocols have been validated with the 100-channel × 100-channel microchip, which generates a total of 10,000 2D pixels and offers a much higher tissue section area coverage.

Spatially resolved sequencing can also be achieved by using oligonucleotide-barcoded arrays, such as Slide-seq[42] and high-definition spatial transcriptomics[43], although only transcriptomics sequencing was demonstrated in these methods. Another study described spatial ATAC[44] coupled with an DNA-barcoded array that contains 5,000 spots with 55 µm spot diameter and 100 µm interspot distance, although this method presents a relatively lower fragment number per spot than our method. More recently, Slide-tags[45] was developed in which individual cell nuclei can be tagged in a oligonucleotide-barcoded bead array and used for transcriptomics or joint transcriptomics and chromatin accessibility profiling. This method has unique advantages in achieving single-nucleus resolution and excellent gene detection sensitivity in transcriptomics applications, although its epigenomics profiling performance (for example, fragment number per nucleus in ATAC-seq) demands further improvements.

Overview of the procedure

Before the spatial-ATAC-RNA-seq or spatial-CUT&Tag-RNA-seq experiment, fresh frozen tissues should be sectioned on poly-L-lysine-coated glass slides (Fig. 1). Polydimethylsiloxane (PDMS) microfluidic array chip fabrication followed by DNA barcode annealing and transposome assembly are described in the 'Reagent setup'.

In spatial-ATAC-RNA-seq, the tissue section is fixed and permeabilized (Fig. 1). Next, transposition is performed in situ, with the Tn5 transposome loaded with a Nextera sequencing adaptor and ligation linker to probe accessible chromatin regions. RT is guided with DNA oligonucleotides (RT primer) that contain a poly-T sequence, a 10-base-pair (bp) UMI sequence and the same ligation linker, to generate cDNA from mRNA molecules as the transcriptomic

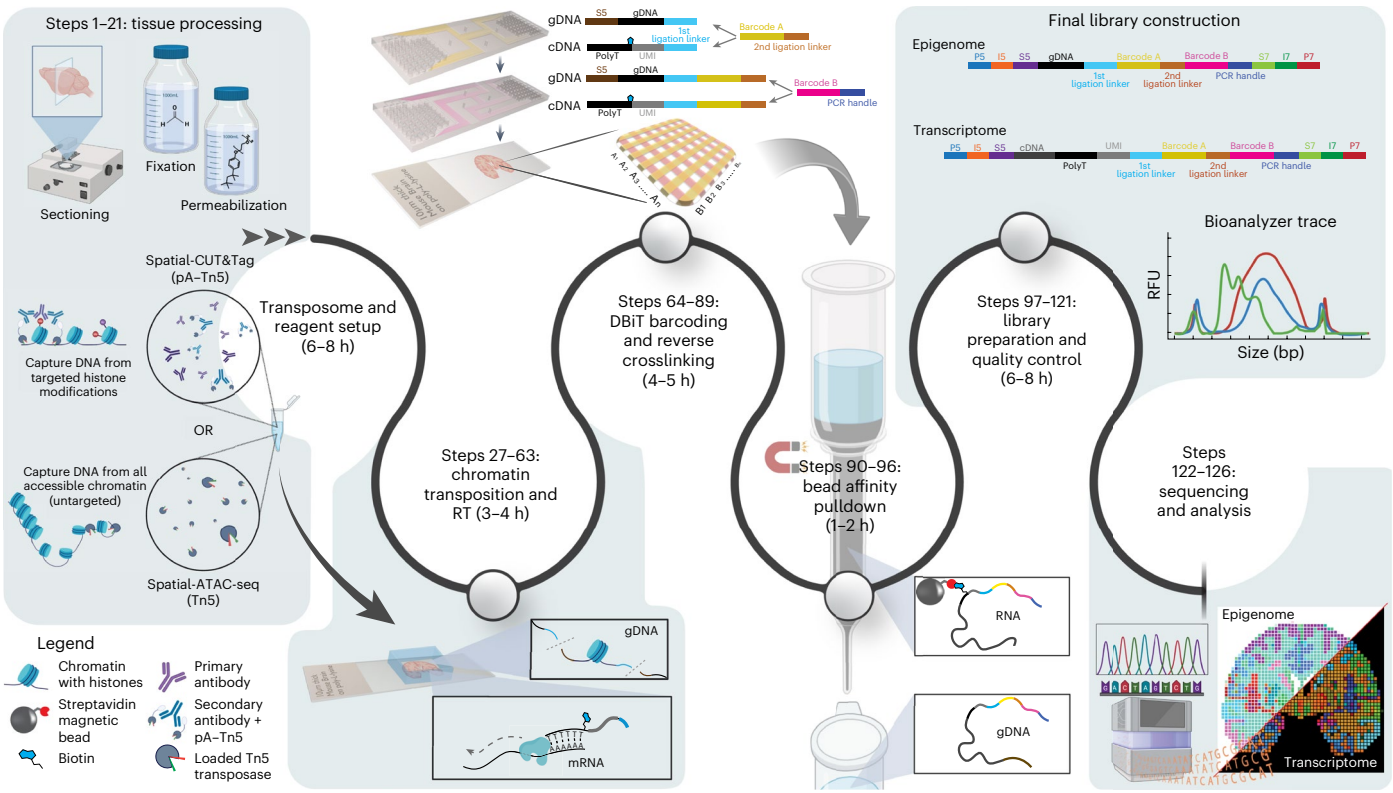

Fig. 1 | Overview of the procedures for spatial-ATAC-RNA-seq and spatial-CUT&Tag-RNA-seq. Both methods perform genome-wide spatially resolved transcriptomic and epigenomic profiling. pA–Tn5 is used in spatial-CUT&Tag-RNA-seq to target specific histone modifications, and Tn5 is used in spatial-ATAC-RNA-seq to capture genomic regions with open chromatin. RFU, relative fluorescence units.

measurement. Then, the first set of spatial barcodes (BC_A) are loaded with the first PDMS microfluidic chip (chip A) and enzymatically ligated with the genomic DNA (gDNA) or cDNA molecules. Next, spatial barcodes (BC_B) are introduced by the second PDMS chip (chip B) with perpendicular microchannels and ligated with the BC_A in situ. gDNA and cDNA are recovered from the chip by reversing the crosslinks. To ensure the separation of the two libraries, the RT primer is modified with an internal biotin, which allows pulling down of cDNA with streptavidin-coupled beads, as previously described[4,5] (Fig. 2). Subsequently, separate libraries are generated from the recovered cDNA and gDNA.

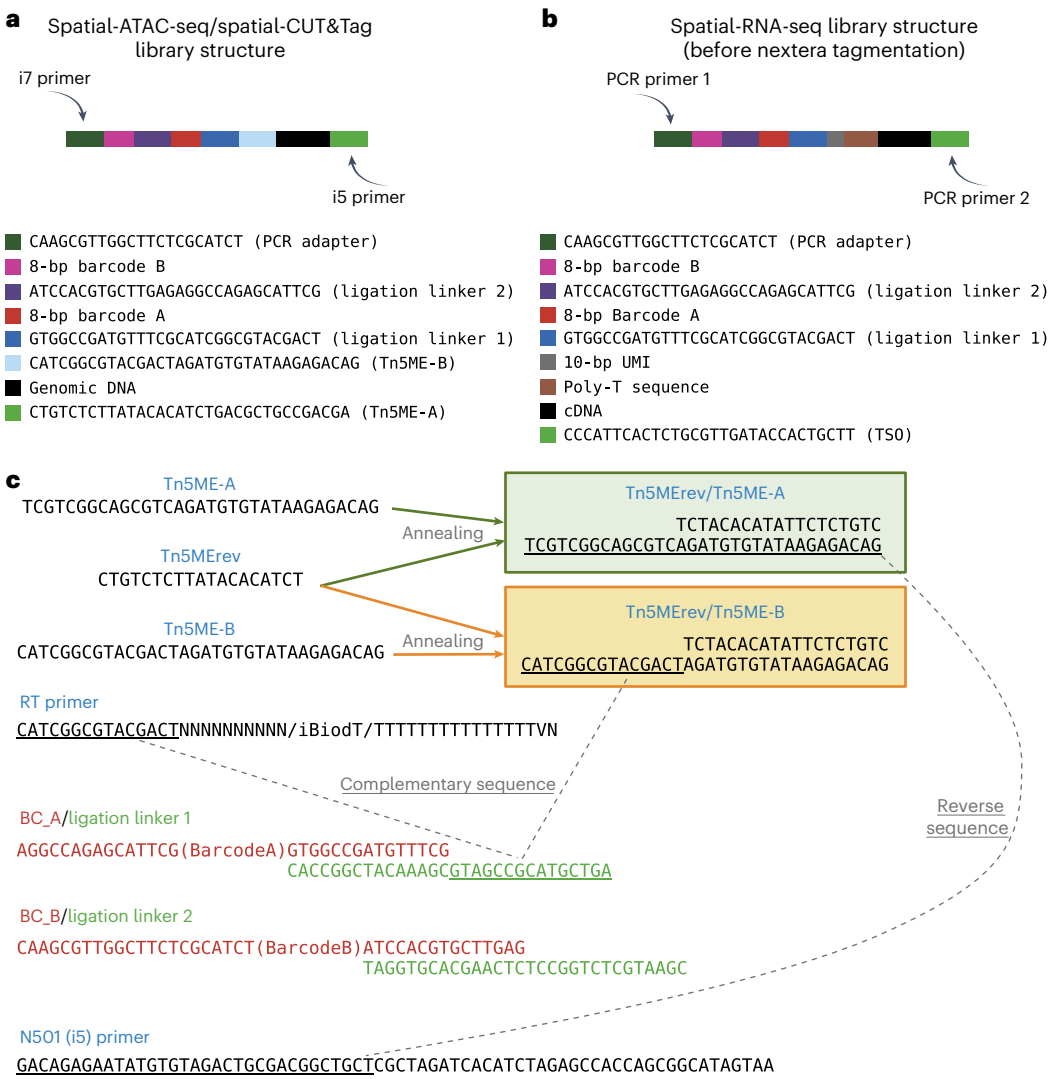

Fig. 2 | Library structure and oligonucleotide primer sequence design. a, In a spatial-ATAC-seq or spatial-CUT&Tag library, after streptavidin-bead affinity pulldown, a pair of i5 and i7 primers, which target the Tn5ME-A sequence and the PCR adapter sequence adjacent to BC_B, respectively, are used for library construction. **b**, In a spatial-RNA-seq library, after template switching, PCR primer 1, which targets the PCR adapter sequence adjacent to BC_B, and PCR primer 2, which targets the TSO sequence, are used for library construction. The library is then prepared using the Nextera Library Preparation kit to generate the final spatial-RNA-seq library. **c**, Tn5MErev has a 19 bp mosaic end sequence that is complementary to both Tn5ME-A and Tn5ME-B so that it can be annealed to both. Spatial barcode A (BC_A) has a sequence complementary to ligation linker 1 and spatial barcode B (BC_B) has a sequence complementary to ligation linker 2. Besides the 19 bp mosaic end sequence, Tn5ME-B contains a sequence that can be ligated with ligation linker 1. This sequence is also identical to the 5′ sequence of the RT primer (sequence underlined in the illustration), which allows co-profiling the two modalities. The sequence of Tn5ME-A is matched with N501 (i5) primers, which allows PCR amplification, with matching sequences underlined in the illustration. iBiodT, internal biotinylated thymidine; V, any nucleotides of A, C or G; N, any nucleotides of A, C, G or T.

In spatial-CUT&Tag-RNA-seq (Fig. 1), the tissue section is fixed and permeabilized with a different buffer from the one used in spatial-ATAC-RNA-seq. The primary antibody targeting a specific histone modification is incubated with the tissue, followed by labeling with a secondary antibody to enhance the tethering of Protein A–Tn5 fusion (pA–Tn5) transposome with the same loaded DNA elements, as described in spatial-ATAC-RNA-seq[21,23]. In situ tagmentation is induced by adding Mg^{2+}-containing tagmentation buffer. Following RT, deterministic barcoding and bead separation are performed in a similar process as used in spatial-ATAC-RNA-seq.

Limitations

The use of the microfluidic channel arrays in spatial-ATAC-RNA-seq and spatial-CUT&Tag-RNA-seq allows co-profiling of genome-wide transcriptomics and epigenomics at near single-cell resolution. However, similar to most current sequencing-based spatially resolved multiomics methods, cells may not be able to segmented if a pixel contains more than one cell, although computational pipelines are available to deconvolute cell type composition of a spot with heterogeneous populations[46]. The current protocol for spatial-CUT&Tag-RNA-seq (or spatial-CUT&Tag) is limited to histone modification profiling, while profiling of chromatin-associated proteins such as transcription factors has not been tested. We direct readers to Farzad et al.[25] to learn more regarding current challenges in spatially resolved ATAC-seq and CUT&Tag technologies. In addition, although chromatin accessibility and histone modifications are two key aspects of epigenetic regulation of gene expression, other epigenetic mechanisms exist, such as DNA methylation, long-range and short-range chromatin interactions, and approaches to spatially resolved profiling of these epigenomic modalities are needed to draw a complete overview of the spatio-temporal epigenetic landscape. Finally, our co-profiling protocol has only been validated on fresh frozen tissue samples. Expanding our methods to include applications on formalin-fixed and paraffin-embedded samples, which are the most readily available sources of disease-relevant human samples, is an essential future direction for translational studies[47].

Experimental design

Tissue sample collection

Fresh tissue samples should be snap-frozen in optimal cutting temperature (OCT) compound as soon as the dissection is completed to avoid degradation of molecules of interest, especially mRNA. When necessary, the tissue quality can be evaluated by measuring the RNA integrity number (RIN) on the extracted bulk RNA, and a RIN score in the range of 7–10 should reflect high-quality RNA preservation[48]. In addition, it is crucial to mount the tissue section on a poly-L-lysine-coated slide, which enhances tissue attachment. For the convenience of PDMS microfluidic chip assembly, it is also important to mount the section in such a way that the ROI lies at the center of the slide.

As an optional design, researchers can prepare serial sections cut from the same tissue block and perform spatial-ATAC-RNA-seq and spatial-CUT&Tag-RNA-seq on adjacent sections for potential alignment of the two datasets. Extra serial sections are also useful for applications such as histology staining, immunofluorescence or in situ hybridization staining, and other high-throughput spatial multiomics methods. After tissue sectioning, the samples can be either used directly for spatial-ATAC-RNA-seq and spatial-CUT&Tag-RNA-seq or stored at −80 °C for future use.

Tn5/pA–Tn5 transposase

The natural naked (unloaded) form of Tn5 transposase is not functional for library generation. To enable tagmentation, the naked Tn5 transposase must be loaded with a pair of annealed oligonucleotide adapters, which contain a double-strand 19 bp mosaic end sequence recognized by the Tn5 transposase and a single-strand transfer sequence[49]. Although a naked Tn5 transposase is commercially available, we note that the loaded Tn5 and pA–Tn5 transposome used in spatial-ATAC-RNA-seq and spatial-CUT&Tag-RNA-seq, respectively, is not commercially available and researchers should perform oligo annealing and Tn5 loading on their own, following the procedure for transposome assembly described in Box 1. Alternatively,

BOX 1

Tn5 and pA–Tn5 transposome assembly

Here, we describe how to perform transposome assembly on naked Tn5 or pA–Tn5 for spatial-ATAC-RNA-seq and spatial-CUT&Tag-RNA-seq applications, respectively. Tn5MErev, Tn5ME-A and Tn5ME-B oligos should be resuspended with annealing buffer (see 'Reagent setup') to a stock concentration of 100 μM.

Procedure for Tn5 transposome assembly

1. In a PCR tube, mix 3 μL 100 μM Tn5MErev with 3 μL 100 μM Tn5ME-A.
2. In another PCR tube, mix 3 μL 100 μM Tn5MErev with 3 μL 100 μM Tn5ME-B.
3. On a thermocycler, incubate the two tubes at 95 °C for 5 min. Then cool down the temperature to 65 °C at a rate of −0.1 °C/s. After incubation at 65 °C for 5 min, cool down the temperature to 4 °C at a rate of −0.1 °C/s.
4. In a PCR tube, mix 5 μL annealed Tn5MErev/Tn5ME-A, 5 μL annealed Tn5Merev/Tn5ME-B and 10 μL unloaded Tn5 transposase (Tn5 stock concentration 2 mg/mL).
5. Gently mix by pipetting and incubate at 23 °C for 30 min.
6. Add 10 μL glycerol, gently mix by pipetting and store at −20 °C for spatial-ATAC-RNA-seq (Tn5 concentration 0.67 mg/mL). The loaded Tn5 can be used within a month.

Procedure for pA–Tn5 transposome assembly

1. In a PCR tube, mix 2.5 μL 100 μM Tn5MErev with 5 μL 100 μM Tn5ME-A.
2. In another PCR tube, mix 2.5 μL 100 μM Tn5MErev with 5 μL 100 μM Tn5ME-B.
3. On a thermocycler, incubate the two tubes at 95 °C for 5 min. Then cool down the temperature to 65 °C at a rate of −0.1 °C/s. After incubation at 65 °C for 5 min, cool down the temperature to 4 °C at a rate of −0.1 °C/s.
4. In a PCR tube, mix 6.25 μL annealed Tn5MErev/Tn5ME-A, 6.25 μL annealed Tn5Merev/Tn5ME-B, and 10 μL unloaded pA–Tn5 transposase (pA–Tn5 stock concentration 3.68 mg/mL).
5. Gently mix by pipetting and incubate at 23 °C for 30 min.
6. Add 12.5 μL glycerol, gently mix by pipetting and store at −20 °C for spatial-CUT&Tag-RNA-seq (pA–Tn5 concentration 1.05 mg/mL). The loaded pA–Tn5 can be used within a month.

laboratories with expertise in protein expression and purification can generate naked Tn5 on their own to reduce reagent cost, as previously described[4,50,51].

Three oligos are used for Tn5 transposome assembly, Tn5MErev, Tn5ME-A and Tn5ME-B (Fig. 2a–c), with sequences available in Supplementary Table 1. Tn5MErev contains the 19 bp mosaic end sequence for Tn5 recognition and both Tn5ME-A and Tn5ME-B contain the reverse complement sequence to this 19 bp sequence so that they can be annealed with Tn5Merev (Fig. 2c). Tn5ME-B also contains a sequence that is complementary to a part of the ligation linker 1 sequence, so that the probed genomic region can be linked to BC_A (Fig. 2c and Supplementary Table 1). Of note, the poly-T oligonucleotide used in RT (RT primer; Supplementary Table 1) shares a sequence with Tn5ME-B that is complementary to the ligation linker 1, which enables reads of both modalities to be linked to BC_A to facilitate co-profiling (Fig. 2c). Tn5ME-A introduces a complementary sequence to i5 primer (Supplementary Table 1), to enable PCR amplification of the probed genomic region in library preparation (Fig. 2a). Spatial-ATAC-RNA-seq and spatial-CUT&Tag-RNA-seq share the same loading DNA elements used in Tn5 and pA–Tn5 transposome assembly.

Spatial barcode annealing

The BC_A oligo contains an 8 bp unique barcode flanked by complementary sequence to ligation linker 2 and ligation link 1 in its 5′ end and 3′ end, respectively (Fig. 2). Similarly, the BC_B oligo contains the i7 primer sequence in its 5′ end followed by an 8 bp unique barcode and a complementary sequence to ligation linker 2. Universal ligation linker 1 and ligation linker 2 are used for annealing with BC_A oligos and BC_B oligos, respectively. The procedure for spatial barcode annealing is described in Box 2. We encourage researchers to freshly prepare annealed spatial barcodes before the spatial-ATAC-RNA-seq or spatial-CUT&Tag-RNA-seq experiment.

Deterministic barcoding microfluidic device

The same PDMS microfluidic chip used in our previous methods, such as DBiT-seq, spatial-ATAC-seq and spatial-CUT&Tag[15,23,24], can also be applied to the spatial-ATAC-RNA-seq and

BOX 2

Spatial barcoding annealing

Here, we describe how to prepare BC_A spatial barcodes annealed with ligation linker 1 and BC_B spatial barcodes annealed with ligation linker 2. All mentioned oligos should be resuspended with nuclease-free H_2O to a stock concentration of 100 μM.

Procedure for BC_A/ligation linker 1 annealing for a 50-channel × 50-channel microfluidic chip

▲ **CRITICAL** In case a 100-channel × 100-channel microfluidic chip is used, prepare a total of 100 PCR tubes and perform the same procedure with 100 different BC_A or BC_B oligos.

1. A total of 50 PCR tubes will be used. Label the tubes from 1 to 50. Alternatively, use a 96-well PCR plate and record the well locations.
2. Add 10 μL 2× annealing buffer (see 'Reagent setup') and 5 μL 100 μM ligation linker 1 to each of the 50 tubes.
   ▲ **CRITICAL STEP** Preparing a master mix containing 2× annealing buffer and ligation linker 1 and distributing 15 μL to each tube is allowed.
3. Add 5 μL 100 μM BC_A barcode to each of the 50 tubes (a unique barcode is added to each tube).
4. On a thermocycler, incubate the tubes at 95 °C for 5 min. Then cool down the temperature from 98 °C to 23 °C at a rate of −0.1 °C/s. After incubation at 12 °C for 5 min, hold the temperature at 4 °C.
   ■ **PAUSE POINT** The annealed BC_A/ligation linker 1 oligos can be stored at 4 °C for over a week or at −20 °C for over 6 months.

Procedure for BC_B/ligation linker 2 annealing for a 50-channel × 50-channel microfluidic chip

1. A total of 50 PCR tubes will be used. Label the tubes from 1 to 50. Alternatively, use a 96-well PCR plate and record the well locations.
2. Add 10 μL 2× annealing buffer (see 'Reagent setup') and 5 μL 100 μM ligation linker 2 to each of the 50 tubes.
3. Add 5 μL 100 μM BC_B barcode uniquely to each of the 50 tubes (a unique barcode is added to each tube).
4. On a thermocycler, incubate the tubes at 95 °C for 5 min. Then cool down the temperature from 98 °C to 23 °C at a rate of −0.1 °C/s. After incubation at 12 °C for 5 min, hold the temperature at 4 °C.
   ■ **PAUSE POINT** The annealed BC_B/ligation linker 2 oligos can be stored at 4 °C for over a week or at −20 °C for over 6 months.

spatial-CUT&Tag-RNA-seq experiments. In brief, the chrome photomasks, with high-resolution computer-aided design (CAD) for the corresponding microfluidic chip printed on the glass substrate, are commercially available[26]. We provide CAD files for both the 50-channel × 50-channel and 100-channel × 100-channel (which allows a higher coverage area) microchips (Supplementary Data) to enable diverse applications depending on researchers' interests. The procedure for PDMS chip preparation is described in Box 3.

Library preparation

As mentioned above, the epigenome and transcriptome libraries are separated with streptavidin-beads. The epigenomic library (i.e., ATAC-seq library of spatial-ATAC-RNA-seq or CUT&Tag library of spatial-CUT&Tag-RNA-seq), is amplified with a pair of i5 and i7 primers directly (Fig. 2a).

The transcriptomic library (that is, RNA-seq), is generated first with template switching, an approach that is used in many transcriptomics sequencing methods[52]. In template switching, we take the advantage of Moloney murine leukemia virus-type reverse transcriptase that adds three nontemplated deoxycytidines to the 3′ end of nascent cDNA that serve as anchors for the template-switching oligo (TSO), which contains three guanosines[53] (Fig. 2b). After template switching, PCR is performed directly on the beads, with PCR primer 1 targeting the universal sequence part of BC_B and PCR primer 2 targeting the TSO sequence (Fig. 2b and Supplementary Table 1). The purified product is then processed for tagmentation with a commercially available Nextera XT DNA Library Preparation kit. Alternatively, researchers can prepare a loaded Tn5 transposome of the same kind for cDNA library tagmentation to reduce reagent cost[54]. Then, PCR is performed on the tagmentation product with a pair of i5 and i7 primers.

Since the epigenomic and transcriptomic libraries can be pooled for next-generation sequencing on the same flow cell, we encourage the use of differently indexed i5/i7 primer pairs during library preparation. Libraries generated from different samples can also be pooled for sequencing.

BOX 3

Preparation of PDMS associated equipment

Here, we describe procedures for preparation of the PDMS microfluidic chips (both chip A and chip B) based on a silicon wafer device mold, as well as preparation of reagent reservoirs made of PDMS. The silicon wafer device mold is made from a silicon wafer and the commercially available chrome photomasks with a standard photolithography technique, and we direct readers to Su et al.[55] for a detailed workflow of silicon wafer preparation along with a video illustration for PDMS microfluidic chip preparation. Here, we also present a video illustration for PDMS reservoir preparation in Supplementary Video 5.

Procedure for PDMS microfluidic chip preparation

1. Place the silicon device wafer mold at the center of a clear 15-cm-diameter Petri dish.
2. In a plastic container (such as a regular disposable plastic cup), mix PDMS elastomer base and curing agent at a 10:1 weight ratio, to a final weight of 50–60 g.
3. Thoroughly mix and pour the mixture onto the silicon device mold.
4. Place the dish in a vacuum desiccator for 30–60 min and remove all bubbles from the mixture with gentle compressed air flow.
5. Incubate the dish at 65–75 °C on an even surface for over 2 h but no longer than 24 h.
6. Carefully cut out the PDMS microfluidic chips with a surgical blade, without damaging the patterned area of the silicon wafer mold surface.
7. Hole punch the inlets/outlets of PDMS microfluidic chips according to the patterned area.
8. Clean the surface of the PDMS chips with Scotch tape.

   ■ **PAUSE POINT** The PDMS chip can be preserved at 20–30 °C for future use (within a year) by covering it with clean Scotch tape.

Procedure for PDMS reservoir preparation

1. In a plastic container (such as a regular disposable plastic cup), mix PDMS elastomer base and curing agent at a 10:1 weight ratio, to a final weight of 50–60 g.
2. Thoroughly mix and pour the mixture onto a clear regular 15-cm-diameter Petri dish.
3. Place the dish in a vacuum desiccator for 30–60 min and remove all bubbles from the mixture.
4. Incubate the dish at 65–75 °C on an even surface for over 2 h.
5. With a surgical blade, cut out several PDMS pieces in a roughly square shape with 20–25 mm slide length.
6. For the large PDMS reservoir, cut out a piece in a roughly square shape with 10–20 mm slide length in the center of the PDMS piece obtained at step 5.
7. For the small PDMS reservoir, cut out a piece in a roughly square shape with 2.5–10 mm slide length in the center of the PDMS piece obtained at step 5.
8. Clean the surface of PDMS reservoirs with Scotch tape.

   ■ **PAUSE POINT** The PDMS reservoir can be preserved at 20–30 °C for future use by covering it with clean Scotch tape.

Materials

Biological materials

Human and mouse brain frozen tissue sections are commercially available from Zyagen. We typically use whole coronal brain sections at a thickness of 10 µm mounted on poly-L-lysine-coated glass slides. Samples should be shipped with dry ice and stored at −80 °C upon receipt.

▲ **CAUTION** For any experiments using animal materials, appropriate national laws and institutional regulatory board guidelines must be followed. The in-house Institutional Animal Care and Use Committee of Zyagen reviewed and approved all protocols related to this work.

▲ **CAUTION** For any experiments using human materials, appropriate national laws and institutional regulatory board guidelines must be followed with informed consent from human subjects. Our original research[26] on human subjects has obtained institutional approval and informed consent and this protocol does not involve new data generated from human materials.

Reagents

General reagents

- Dulbecco's phosphate-buffered saline (DPBS) (Gibco, cat no. 14190144)
- Phosphate-buffered saline (PBS) (Gibco, cat no. 10010023)
- 16% (wt/vol) Formaldehyde solution (Thermo Fisher Scientific, cat no. PI28906)

  ▲ **CAUTION** Avoid inhalation and skin contact. Dispose unused solution following proper hazardous waste procedures.

- 10% (wt/vol) NP-40 detergent solution (Thermo Scientific, cat. no. 28324)
  ▲ **CAUTION** NP-40 can cause skin and eye irritation. Wear protective gloves and clothing while working with NP-40 solution and seal the bottle after use.
- Proteinase K (Thermo Fisher Scientific, cat. no. EO0491)
- Maxima H Minus Reverse Transcriptase (Thermo Fisher Scientific, cat. no. EP0752)
- 10 mM dNTP Mix (Thermo Fisher Scientific, cat. no. R0192)
- EvaGreen dye (Biotium, cat. no. 31000-T)
- 5 M NaCl (Thermo Fisher Scientific, cat no. AM9760G)
- 1 M $MgCl_2$ (Thermo Fisher Scientific, cat no. AM9530G)
- Nuclease-free water (Invitrogen, cat. no. AM9932)
- SUPERase In RNase Inhibitor (Invitrogen, cat. no. AM2694)
- Dynabeads MyOne Streptavidin C1 (Invitrogen, cat. no. 65001)
- 1 M Tris–HCl pH 8 (Invitrogen, cat no. 15568025)
- 1 M Tris–HCl pH 7.4 (Rockland, cat no. MB-002)
- Enzymatics RNase Inhibitor (Qiagen, cat. no. Y9240L)
- KAPA HiFi HotStart ReadyMix (Roche, cat. no. KK2601)
- 20 mg/mL (2% (wt/vol) in DMSO) Digitonin (Promega, cat. no. G9441)
  ▲ **CAUTION** Digitonin may cause damage to organs through prolonged or repeated exposure. Wear protective gloves and clothing while working with it.
- 10% (wt/vol) Sodium dodecyl sulfate (SDS) (Sigma-Aldrich, cat. no.71736)
- Glycine (Sigma-Aldrich, cat. no. 50046)
- Triton X-100 (Sigma-Aldrich, cat. no. T8787)
- Bovine Serum albumin (BSA) (Sigma-Aldrich, cat. no. A8806)
- 20% (wt/vol) Ficoll PM-400 (Sigma-Aldrich, cat. no. F5415)
- 10% (wt/vol) Tween-20 (Millipore-Sigma, cat. no. 11332465001)
- 0.5 M EDTA Solution pH 8.0 (American Bio, cat. no. AB00502)
- T4 DNA Ligase (New England Biolabs, cat. no. M0202L)
- T4 DNA Ligase Reaction Buffer (New England Biolabs, cat. no. B0202S)
- NEBuffer 3.1 (New England Biolabs, cat. no. B7203S)
- NEBNext High-Fidelity 2× PCR Master Mix (New England Biolabs, cat. no. M0541L)
- Ampure XP beads (Beckman Coulter, cat. no. A63880)
  ▲ **CRITICAL STEP** SPRIselect beads (Beckman Coulter, cat. no. B23317) can be used as an alternative reagent.
- DNA Clean & Concentrator-5 (Zymo Research, cat. no. D4014)
- Nextera XT DNA Library Preparation kit (Illumina, cat. no. FC-131-1024)
- TapeStation High Sensitivity D5000 ScreenTape (Agilent, cat. no. 5067-5592)
- TapeStation High Sensitivity D5000 Reagents (Agilent, cat. no. 5067-5593)
- Oligonucleotides (IDT, see Supplementary Table 1 for sequences)
  ▲ **CRITICAL STEP** All oligos are ordered in the lyophilized form. Pay attention to the buffer used for resuspension listed in the table.
  ▲ **CRITICAL STEP** TSO primers may degrade if not stored properly. Aliquoting TSO primers after initial preparation of the stock solution (20 µL per aliquot) and storing the aliquots at −20 °C is recommended.

Reagents specific to spatial-ATAC-RNA-seq

- 2× Tagmentation buffer (Diagenode, cat. no. C01019043)
- Tn5 Transposase, unloaded (Diagenode, cat. no. C01070010)

Reagents specific to spatial-CUT&Tag-RNA-seq

- 1 M HEPES pH 7.5 (Boston BioProducts, cat. no. BBH-75-250)
- EDTA-free Protease Inhibitor Cocktail (Millipore Sigma, cat. no.11873580001)
- Spermidine (Sigma-Aldrich, cat. no. S0266)
- α-H3K27me3 antibody (Cell Signaling Technology, cat no. 9733, https://scicrunch.org/resolver/AB_2616029); (http://antibodyregistry.org/AB_2616029)

- α-H3K4me3 antibody (Active Motif, cat. no. 39159, https://scicrunch.org/resolver/AB_2615077); (http://antibodyregistry.org/AB_2615077)
- α-H3K27ac antibody (Abcam, cat. no. ab177178, https://scicrunch.org/resolver/AB_2828007); (http://antibodyregistry.org/AB_2828007)
- Guinea Pig anti-Rabbit IgG Secondary antibody (Antibodies-Online, cat. no. ABIN101961, https://scicrunch.org/resolver/AB_10775589); (http://antibodyregistry.org/AB_10775589)
- pA-Tn5 Transposase, unloaded (Diagenode, cat. no. C01070002)

Equipment

- Hole punching machine (SCHMIDT Manual Press or equivalent)
- 100 mm polished silicon wafer (WaferPro, cat. no. C04004)
- Vacuum desiccator (Sigma, cat. no. Z119024)
- Surgical Blade Handles (Aspen Surgical, cat. no. 371030)
- SYLGARD 184 Silicone Elastomer Base and Curing Agent (Dow Corning, cat. no. 4019862)
- PDMS microfluidic chips (Box 3)
- Large and small PDMS reservoirs (Box 3)
- Poly-L-lysine-coated glass slide (Electron Microscopy Sciences, cat. no. 63478-AS)
- Scotch Magic Tape (Scotch, cat. no. 810)
- Microscopy. We use EVOS FL Auto imaging system (Invitrogen, cat. no. AMF7000) to scan and image the tissue section, typically at a magnification of 10×. Other optical microscopes with whole slide scanning functions can also be used for this purpose
- Custom-designed acrylic clamp with screws[55]
- Homemade vacuum system for fluid flow in the microfluidic channels[55]
- 96-well PCR plates (BioRad, cat. no. MLL9601)
- 1.5 mL DNA LoBind Microcentrifuge Tubes (Eppendorf, cat. no. 13698791)
- Microseal PCR plate sealing film (BioRad, cat. no. MSB1001)
- TapeStation 4150 instrument (Agilent, cat. no. G2992A)
- T100 Thermal Cycler (BioRad, cat. no. 1861096)
- CFX Connect Real-Time PCR Detection System (BioRad, cat. no. 1855201)
- Regular laboratory equipment, including vortex mixer, microcentrifuge, 0.2 mL PCR tube strips, 15 mL and 50 mL conical tubes, single-channel and multi-channel pipettes, 15 cm diameter Petri dish
- Tube Revolver Rotator (Thermo Scientific, cat. no. 88881001)
- Fisherbrand Heat/Cool Thermal Mixer II (Thermo Scientific, cat. no. 15-600-330)

Software or computational framework

- Cell Ranger ATAC[56] v.1.2 (10X Genomics)
- ST pipeline[57] v.1.7.2
- A pipeline for spatially resolved transcriptomics data processing: https://github.com/rongfan8/DBiT-seq
- A pipeline for spatial-ATAC-seq data processing: https://github.com/dyxmvp/Spatial_ATAC-seq
- A pipeline for spatial-CUT&Tag data processing: https://github.com/dyxmvp/spatial-CUT-Tag
- Adobe Illustrator v.25.4.3 for raw image processing, with a manual described in https://github.com/rongfan8/DBiT-seq
- Seurat[7] v4.1
- Signac[58] v1.8
- ArchR[59] v1.0.1
- SnapATAC2[60] v2.6.0

Reagent setup

General reagents

Annealing buffer

This buffer consists of Tris–HCl (pH 8.0) at a final concentration of 40 mM and NaCl at a final concentration of 50 mM in nuclease-free H_2O. This buffer can be stored at 20–25 °C for over 6 months.

2× Annealing buffer
This buffer consists of 20 mM Tris-HCl (pH 8.0), 100 mM NaCl and 2 mM EDTA in nuclease-free H_2O. We recommend preparing this fresh buffer prepared every time.

1% (wt/vol) Digitonin
Dilute 2% (wt/vol) Digitonin with an equal volume of nuclease-free H_2O to a 1% (wt/vol) stock solution. 1% (wt/vol) Digitonin can be stored at −20 °C for up to 6 months. Avoid more than five freeze-thaw cycles.

40 mM EDTA
Add 80 µL 0.5M EDTA to 920 µL nuclease-free H_2O. This buffer can be stored at 20–25 °C for over 6 months.

0.5× DPBS
Mix 25 mL 1× DPBS with 25 mL nuclease-free H_2O. This buffer can be stored at 20–25 °C for over a year.

1× PBS–RI
Add 5 µL 40 U/µL Enzymatics RNase Inhibitor into 4 mL 1× PBS so that the final concentration is 0.05 U/µL. The buffer should be freshly prepared each time and stored on ice until use.

0.5× PBS–RI
Mix 0.5 mL 1× PBS–RI with 0.5 mL nuclease-free H_2O. The buffer should be freshly prepared each time and stored on ice until use.

0.2% (wt/v) formaldehyde
Prepare 0.2% (wt/v) formaldehyde by adding 10 µL 16% (wt/vol) formaldehyde stock to 790 µL 1× PBS–RI. The buffer should be freshly prepared each time.

1.25 M glycine
Dissolve 0.94 g of glycine powder in 10 mL of 1× PBS. The solution can be stored at 4 °C for over 6 months.

1× NEB buffer 3.1
Prepare 1.5 mL 1× NEB buffer 3.1 by adding 150 µL 10× NEB buffer 3.1 to 1,350 µL nuclease-free H_2O, supplemented with 10 µL Enzymatics RNase Inhibitor. The buffer should be freshly prepared each time and stored on ice until use.

RT mix
Prepare the RT mix with the recipe below. The buffer should be freshly prepared each time and stored on ice until use.

Reagent	Volume (µL)	Stock concentration	Final concentration
5× RT buffer	12.5	5×	1×
Nuclease-free H_2O	4.5	–	–
Enzymatics RNase Inhibitor	0.4	40 U/µL	0.256 U/µL
SUPERase In RNase Inhibitor	0.8	20 U/µL	0.256 U/µL
dNTPs	3.1	10 mM	0.5 mM
Maxima H Minus Reverse Transcriptase	6.2	200 U/µL	20 U/µL
RT primer	10	100 µM	16 µM
0.5× PBS–RI	25	–	–
Total	62.5	–	–

In situ ligation mix

Prepare the in situ ligation mix with the recipe below. The buffer should be freshly prepared each time and stored on ice until use.

Reagent	Volume (μL)	Stock concentration	Final concentration
T4 ligase buffer	27	5×	1×
5% Triton X-100	5.4	10 mM	0.5 mM
T4 DNA ligase	11	400 U/μL	19 U/μL
Nuclease-free H_2O	72.4	–	–
1× NEB 3.1	115.8	–	–
Total	231.6	–	–

Reverse crosslinking buffer

Prepare the reverse crosslinking buffer with the recipe below. The buffer should be freshly prepared each time and stored on ice until use.

Reagent	Volume (μL)	Stock concentration	Final concentration
Tris–HCl (pH 8.0)	7.5	1 M	50 mM
EDTA	0.3	0.5 M	1 mM
SDS	15	10% (wt/vol)	1% (wt/vol)
NaCl	6	5 M	200 mM
Proteinase K	3	20 mg/mL	0.4 mg/mL
Nuclease-free H_2O	118.2	–	–
Total	150	–	–

1× B&W-T buffer

This buffer consists of Tris–HCl (pH 8.0) at a final concentration of 5 mM, NaCl at a final concentration of 1 M, EDTA at a final concentration of 0.5 mM, Tween 20 at a final concentration of 0.05% (vol/vol) in nuclease-free H_2O. This buffer can be stored at 4 °C for over 6 months.

2× B&W buffer

This buffer consists of Tris–HCl (pH 8.0) at a final concentration of 10 mM, NaCl at a final concentration of 2 M, EDTA at a final concentration of 1 mM in nuclease-free H_2O. This buffer can be stored at 4 °C for over 6 months.

TSO mix

Prepare TSO mix with the recipe below. The buffer should be freshly prepared each time and stored on ice until use.

Reagent	Volume (μl)	Stock concentration	Final concentration
5× RT buffer	44	5×	1×
Nuclease-free H_2O	88	–	–
20% Ficoll PM-400	44	20% (wt/vol)	4% (wt/vol)
Enzymatics RNase Inhibitor	5.5	40 U/μL	1 U/μL
dNTPs	22	10 mM	1 mM
TSO primer	5.5	100 μM	2.5 μM
Maxima H Minus Reverse Transcriptase	11	200 U/μL	10 U/μL
Total	220	–	–

Reagents specific to spatial-ATAC-RNA-seq

5% (wt/vol) BSA

Prepare 10 mL 5% (wt/vol) BSA by dissolving BSA in PBS. The buffer can be stored at 4 °C and used within 3 months.

10× ATAC permeabilization buffer

Prepare 1 mL 10× ATAC permeabilization buffer with the recipe below. The buffer should be freshly prepared each time.

Reagent	Volume (μL)	Stock concentration	Final concentration
Tris–HCl (pH 7.4)	10	1 M	10 mM
NaCl	2	5 M	10 mM
$MgCl_2$	3	1 M	3 mM
Tween-20	10	10% (vol/vol)	0.1% (vol/vol)
NP-40	10	10%	0.1%
Digitonin	10	1% (wt/vol)	0.01% (wt/vol)
BSA	200	5% (wt/vol)	1% (wt/vol)
Nuclease-free H_2O	755	–	–
Total	1,000		

ATAC permeabilization dilution buffer

Prepare 2 mL ATAC permeabilization dilution buffer with the recipe below. The buffer should be freshly prepared each time.

Reagent	Volume (μL)	Stock concentration	Final concentration
Tris–HCl (pH 7.4)	20	1 M	10 mM
NaCl	4	5 M	10 mM
$MgCl_2$	6	1 M	3 mM
BSA	400	5% (wt/vol)	1% (wt/vol)
Nuclease-free H_2O	1,570	–	–
Total	2,000		

ATAC wash buffer

Prepare 2 mL ATAC wash buffer with the recipe below. The buffer should be freshly prepared each time.

Reagent	Volume (μL)	Stock concentration	Final concentration
Tris–HCl (pH 7.4)	20	1 M	10 mM
NaCl	4	5 M	10 mM
$MgCl_2$	6	1 M	3 mM
BSA	400	5% (wt/vol)	1% (wt/vol)
Tween-20	20	10% (vol/vol)	0.1% (vol/vol)
Nuclease-free H_2O	1,550	–	–
Total	2,000		

ATAC transposition mix

Prepare the buffer by mixing 50 μL 2× tagmentation buffer (Diagenode), 33 μL 1× PBS–RI, 1 μL 10% (vol/vol) Tween-20, 1 μL 1% (wt/vol) Digitonin, 5 μL loaded Tn5 transposome (Box 1) and 10 μL nuclease-free H_2O. The total volume is 100 μL. The final Tn5 concentration is 0.033 mg/mL. The buffer should be freshly prepared each time and stored on ice.

Reagents specific to spatial-CUT&Tag-RNA-seq

2 M Spermidine stock solution

Prepare by dissolving 1 g Spermidine into 3.44 mL nuclease-free H_2O. Prepare 500 μL aliquots and store at −20 °C for a month.

CUT&Tag wash buffer

Prepare 50 mL of buffer, which consists of 20 mM HEPES pH 7.5, 150 mM NaCl, 0.5 mM spermidine and one tablet of protease inhibitor cocktail in nuclease-free H_2O. The buffer should be stored on ice or at 4 °C and used within 2 d.

NP40–digitonin wash buffer

Prepare the buffer with the recipe below. The buffer should be stored on ice or at 4 °C and used within 1 d.

Reagent	Volume (µL)	Stock concentration	Final concentration
Digitonin	40	1% (wt/vol)	0.01% (wt/vol)
NP-40	4	10% (vol/vol)	0.01% (vol/vol)
CUT&Tag wash buffer	3,956	–	–
Total	4,000		

Antibody buffer

Prepare ~2.5 mL antibody buffer by mixing 2.5 mL NP40–digitonin wash buffer with 0.5 µL 5% (wt/vol) BSA and 10 µL 0.5 M EDTA. The buffer should be freshly prepared each time and stored on ice until use. Mix 125 µL antibody buffer with 1 µL SUPERase In RNase Inhibitor for the primary antibody binding step.

300-Wash buffer

2.5 mL 300-wash buffer can be prepared by adding 75 µL 5 M NaCl to 2.425 mL CUT&Tag wash buffer, with a final concentration of NaCl as 300 mM. The buffer should be freshly prepared each time and stored on ice until use. The 300-wash buffer should be supplemented with 0.5% (vol/vol) SUPERase In RNase Inhibitor before use.

Equipment setup

PDMS microfluidic devices and PDMS reservoirs need to be fabricated following the step-by-step procedure detailed in Box 3.

Procedure

Tissue fixation

● **TIMING 30–45 min**

▲ **CRITICAL** These steps are required for both spatial-ATAC-RNA-seq and spatial-CUT&Tag-RNA-seq experiments.

1. Freshly prepare 1× PBS–RI and 0.2% (vol/vol) formaldehyde ('Reagent setup').
   ▲ **CAUTION** Avoid inhalation and skin contact when working with formaldehyde. Dispose of unused solution following proper hazardous waste procedures.
2. If the annealed spatial barcodes (BC_A annealed with ligation linker 1; BC_B annealed with ligation linker 2) are stored at −20 °C, thaw the barcodes at 4 °C, which takes 1–2 h.
3. Warm up the slides with frozen OCT tissue section at 20–25 °C for 15 min.
4. Fix the tissue section by adding 200–500 µL 0.2% (vol/vol) formaldehyde.
   - Incubate at 20–25 °C for 5 min
   - Make sure to cover all the OCT regions with formaldehyde
   - After fixation, pipette off the residual solution and discard

   ▲ **CRITICAL STEP** Fixation is mandatory to avoid RNA diffusion or degradation in the subsequent procedures before RT. We typically use 0.2% (vol/vol) formaldehyde for mouse and human brain sections of 10 µm thickness. Optimization of formaldehyde concentration may be needed for different sample types and different section thicknesses.

5. Quench fixation by adding 500–1,000 µL 1.25 M glycine.
   - Incubate at 20–25 °C for 5 min
   - Make sure to cover all the formaldehyde-covered regions with glycine
6. After incubation with glycine, pipette off the residual solution and discard. Proceed to Step 7 for Spatial-ATAC-RNA-seq or Step 20 for Spatial-CUT&Tag-RNA-seq.
   ◆ **TROUBLESHOOTING**

Tissue permeabilization (ATAC-seq)

● **TIMING 30–45 min**

▲ **CRITICAL** Permeabilization is required for both spatial-ATAC-RNA-seq and spatial-CUT&Tag-RNA-seq experiments; however, different permeabilization and wash buffer recipes are used for the two procedures (Fig. 3 and 'Reagent setup'). Perform procedures of this section for the spatial-ATAC-RNA-seq experiment only.

7. Briefly wash the tissue section two times with 1 mL 0.5× DPBS supplemented with 5 µL SUPERase In RNase Inhibitor and 5 µL Enzymatics RNase Inhibitor. Wash by pipetting the solution to cover the tissue section and then pipette and discard the solution.
8. Dip the slide in deionized water (diH_2O) for 1 min and dry the slide with compressed air.
   ◆ **TROUBLESHOOTING**
9. Attach the large PDMS reservoir to the tissue section and apply the acrylic clamp[55] (Fig. 3 and Supplementary Video 1).
   ▲ **CRITICAL STEP** Make sure the tissue ROI is accessible to reagents.
10. Freshly prepare 10× ATAC permeabilization buffer, ATAC permeabilization dilution buffer and ATAC wash buffer (see 'Reagent setup').
11. Prepare 1× ATAC permeabilization buffer by mixing 100 µL 10× ATAC permeabilization buffer with 900 µL ATAC permeabilization dilution buffer and 2.5 µL SUPERase In RNase Inhibitor and 1.25 µL Enzymatics RNase Inhibitor.
12. Add 200–500 µL 1× ATAC permeabilization buffer into the reservoir.
13. Permeabilize the tissue on the slide by incubating it at 20–25 °C for 15 min.
14. During the reaction, prepare ATAC transposition mix (see 'Reagent setup'), which will be used in the subsequent procedure.
15. Carefully aspirate reagents in the reservoir after the reaction.
    ◆ **TROUBLESHOOTING**
16. Add 200–500 µL ATAC wash buffer into the reservoir and incubate at 20–25 °C for 5 min.
17. Carefully aspirate the liquid in the reservoir after the incubation and discard.
18. Wash the tissue with 200–500 µL ATAC wash buffer for 1 min.
19. Carefully aspirate the liquid in the reservoir, discard it and proceed to chromatin transposition (Step 27).

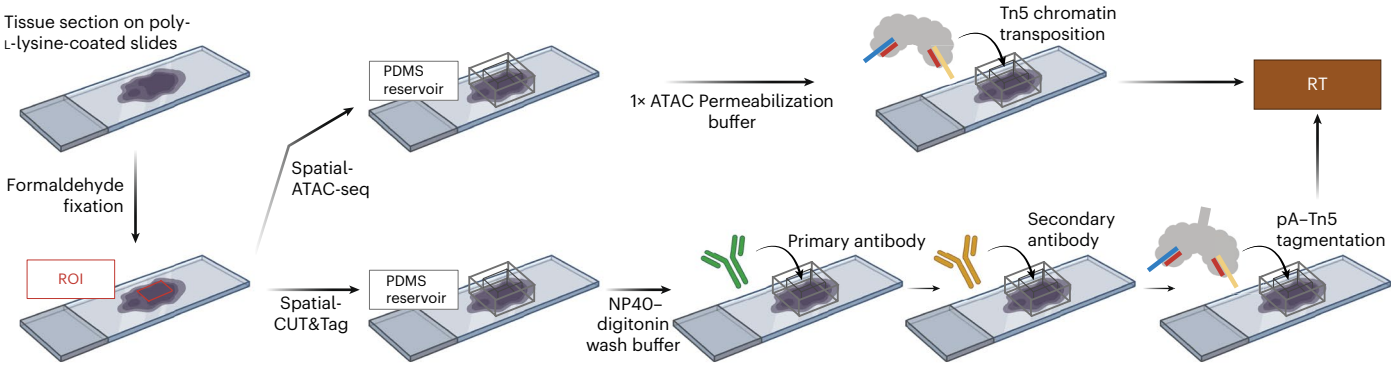

Fig. 3 | Procedures for tissue fixation, permeabilization and Tn5 or pA-Tn5 tagmentation. The tissue is mounted on a poly-L-lysine-coated glass slides without frosted regions. A large PDMS reservoir should be applied over the tissue ROI after tissue fixation. The spatial-ATAC-RNA-seq and spatial-CUT&Tag-RNA-seq procedures use wash and permeabilization buffers with different recipes. Tn5 is used for in situ chromatin transposition in spatial-ATAC-RNA-seq. In spatial-CUT&Tag-RNA-seq, the section is incubated with a primary antibody targeting specific histone modification, a secondary antibody and pA–Tn5 for tagmentation.

Tissue permeabilization (CUT&Tag)

● **TIMING 10–20 min**

▲ **CRITICAL** Perform procedures of this section for the spatial-CUT&Tag-RNA-seq experiment only.

20. Freshly prepare CUT&Tag wash buffer, NP40–digitonin wash buffer and antibody buffer (see 'Reagent setup').
21. Wash the tissue section from Step 6 two times with 1 mL CUT&Tag wash buffer supplemented with 5 µL SUPERase In RNase Inhibitor and 5 µL Enzymatics RNase Inhibitor.
22. Dip the slide in diH_2O for 1 min and dry the slide with compressed air.
    ◆ **TROUBLESHOOTING**
23. Attach the large PDMS reservoir to the tissue section and apply the acrylic clamp[55] (Fig. 3 and Supplementary Video 1).
    ▲ **CRITICAL STEP** Make sure the tissue ROI is accessible to reagents.
24. Add 200–500 µL NP40–digitonin wash buffer into the reservoir.
25. Permeabilize the tissue by incubating it at 20–25 °C for 5 min.
26. Carefully aspirate the liquid in the reservoir, discard it and proceed antibody binding (Step 38).

Chromatin transposition (ATAC-seq)

● **TIMING 60–75 min**

▲ **CRITICAL** Perform procedures of this section for the spatial-ATAC-RNA-seq experiment only. A video illustration for Steps 27–37 is available in Supplementary Video 2. Make sure that 1× PBS–RI (containing RNase inhibitors) is used for preparation of ATAC transposition mix (see 'Reagent setup') to avoid RNA degradation.

27. Add 100 µL ATAC transposition mix with the loaded Tn5 transposome into the reservoir (from Step 19).
28. Place the slide in a humidified chamber (a lidded container with a wet tissue placed at the bottom) and incubate at 37 °C for 30–45 min.
    ▲ **CRITICAL STEP** We typically perform ATAC-seq for 30 min on mouse and human brain sections of 10 µm thickness. For these samples, the incubation time should not exceed 45 min to avoid high background of tagmentation. Optimization of incubation time may be needed for different sample types and different section thicknesses.
29. Carefully aspirate the liquid in the reservoir and discard.
30. Stop chromatin transposition by adding 200 µL 40 mM EDTA supplemented with 0.5 µL SUPERase In RNase Inhibitor.
31. Pipette up and down ten times after adding EDTA solution.
32. Incubate at 20–25 °C for 5 min.
33. Carefully aspirate the liquid in the reservoir and discard.
34. To wash the tissue section, add 200 µL 0.5× PBS–RI to the reservoir.
35. Incubate at 20–25 °C for 1 min.
36. Carefully aspirate the liquid in the reservoir and discard.
37. Repeat the 0.5× PBS–RI wash (Steps 34–36) once.

Antibody binding and tagmentation (CUT&Tag)

● **TIMING Overnight incubation >3–4 h**

▲ **CRITICAL** Perform procedures of this section for the spatial-CUT&Tag-RNA-seq experiment only. A video illustration for Steps 38–55 is available in Supplementary Video 2. Make sure that antibody buffer and 300-wash buffer (see 'Reagent setup') are supplemented with RNase inhibitors before use.

38. Mix 2.5 µL primary antibody with 125 µL antibody buffer and add to the large PDMS reservoir (from Step 26).
    ◆ **TROUBLESHOOTING**
39. Place the slide in a humidified chamber and incubate the tissue with primary antibody at 4 °C overnight.
40. Carefully aspirate the liquid in the reservoir and discard.
41. Wash the slide with NP40–digitonin wash buffer three times for 5 min each.

42. Mix 2.5 µL secondary antibody with 125 µL NP40–digitonin wash buffer, supplemented with 1 µL SUPERase In RNase Inhibitor, and add the solution to the PDMS reservoir.
43. Place the slide in a humidified chamber and incubate at 20–25 °C for 30 min.
44. Once the incubation is complete, carefully aspirate the liquid in the reservoir and discard.
45. Wash the tissue for three times with 300 µL CUT&Tag wash buffer at 20–25 °C for 5 min per wash.
    - Pipette up and down ten times after adding the buffer
    - Carefully aspirate the liquid in the reservoir after each wash and discard
    - During the washes, freshly prepare 300-wash buffer (see 'Reagent setup')
46. Mix 2.5 µL loaded pA–Tn5 transposome (Box 1) with 250 µL 300-wash buffer and add the solution to the PDMS reservoir. The concentration of pA–Tn5 in the reaction mix is ~10.4 µg/mL.
47. Place the slide in a humidified chamber and incubate at 20–25 °C for 1 h.
48. Carefully aspirate the liquid in the reservoir after the reaction and discard.
49. Wash the tissue on the slide two to three times with 300 µL 300-wash buffer at 20–25 °C for 5 min per wash.
    - Pipette up and down ten times after adding the buffer
    - Carefully aspirate the wash buffer after each wash and discard
50. Mix 300 µL 300-wash buffer with 3 µL 1 M $MgCl_2$ (at a final concentration of 10 mM) and add to the PDMS reservoir.
    ▲ **CRITICAL STEP** pA–Tn5 is activated in the presence of Mg^{2+} to enable tagmentation.
51. Place the slide in a humidified chamber and incubate at 37 °C for 1 h.
52. Carefully aspirate the liquid in the reservoir after the incubation and discard.
53. Stop tagmentation by adding 200 µL 40 mM EDTA supplemented with 0.5 µL SUPERase In RNase Inhibitor.
    - Pipette up and down ten times after adding the EDTA solution
    - Incubate at 20–25 °C for 5 min
    - Carefully aspirate the solution and discard it
54. Wash the tissue section:
    - Add 200 µL 0.5× PBS–RI to the reservoir
    - Incubate at 20–25 °C for 1 min
    - Carefully aspirate the solution and discard it
55. Repeat the 0.5× PBS–RI wash once.

RT

● **TIMING 120–150 min**

▲ **CRITICAL** This procedure is required for both spatial-ATAC-RNA-seq and spatial-CUT&Tag-RNA-seq experiments.

56. Prepare the RT mix (see 'Reagent setup').
57. Load the RT mix onto the tissue section from Step 37 or 55 (with a PDMS reservoir attached) and perform RT:
    - Place the slide in a humidified chamber
    - Incubate at 20–25 °C for 30 min, and 42 °C for 90 min
    - During the reaction, prepare 1× NEB buffer 3.1 (see 'Reagent setup'), which will be used in Step 60
    - Carefully aspirate the liquid in the reservoir and discard
58. Carefully release the acrylic clamp and remove the PDMS reservoir.
59. Quickly dip the slide in 1× PBS for 30 s.
60. Add 300–500 µL 1× NEB buffer 3.1 onto the section.
61. Incubate at 20–25 °C for 5 min and carefully aspirate and discard the buffer.
62. Dip the slide in diH_2O for 1 min and dry the slide with compressed air.
63. Scan the tissue section with the EVOS FL auto imaging system (10× objective; bright field).
    ◆ **TROUBLESHOOTING**

Deterministic barcoding in tissue (barcode A)

● **TIMING 45–60 min**

▲ **CRITICAL** This procedure is required for both spatial-ATAC-RNA-seq and spatial-CUT&Tag-RNA-seq experiments.

64. Place PDMS microfluidic chip A onto the tissue slide, ensuring that the reservoir aligns with the tissue ROI (Fig. 4 and Supplementary Video 3).
    - Make sure the tissue section on the slide is fully dry before applying the PDMS chip
    - Clamp the PDMS chip and the slide tightly with the acrylic clamp described above

    ▲ **CRITICAL STEP** The channels of the PDMS microfluidic chip should be kept free of dust to ensure smooth flow of solution.
65. Prepare the in situ ligation mix (see 'Reagent setup').

    ▲ **CRITICAL STEP** For convenience, this protocol is written for the use of 50-channel × 50-channel microchips. Adjust the reagent volume accordingly if using 100-channel × 100-channel microchips.
66. In a 96-well plate, for each well, add 4 µL in situ ligation mix.
67. Add 1 µL annealed BC_A/ligation linker 1 oligos (Box 2) to each well. Record the positions where the uniquely indexed barcodes are added to each well.
68. Load 5 µL barcode mixture from each well of the 96-well plate to each inlet of the PDMS microfluidic chip, following the sequence arrangement shown in Fig. 4.

    ▲ **CRITICAL STEP** Make sure the mixtures containing the uniquely index barcodes are added to the correct inlet positions since the barcodes correspond to the *x*-axis coordinates used in data analysis.
69. Apply a vacuum cap on the outlet reservoir and let the barcode mixture flow slowly through the channels for 5 min.

    ▲ **CRITICAL STEP** Make sure all the channels over the tissue ROI are filled with the barcode mixture.

    ▲ **CRITICAL STEP** Start with a gentle air pressure and increase the pressure gradually. Never apply vacuum to the channels with high pressure to avoid leakage and tissue detachment.
70. Place the slide with the PDMS chip in a humidified chamber and incubate at 37 °C for 30 min.
71. Suck solution out of the inlets and outlets by applying a vacuum aspiration system.
72. Carefully release the acrylic clamp and remove the PDMS microfluidic chip.

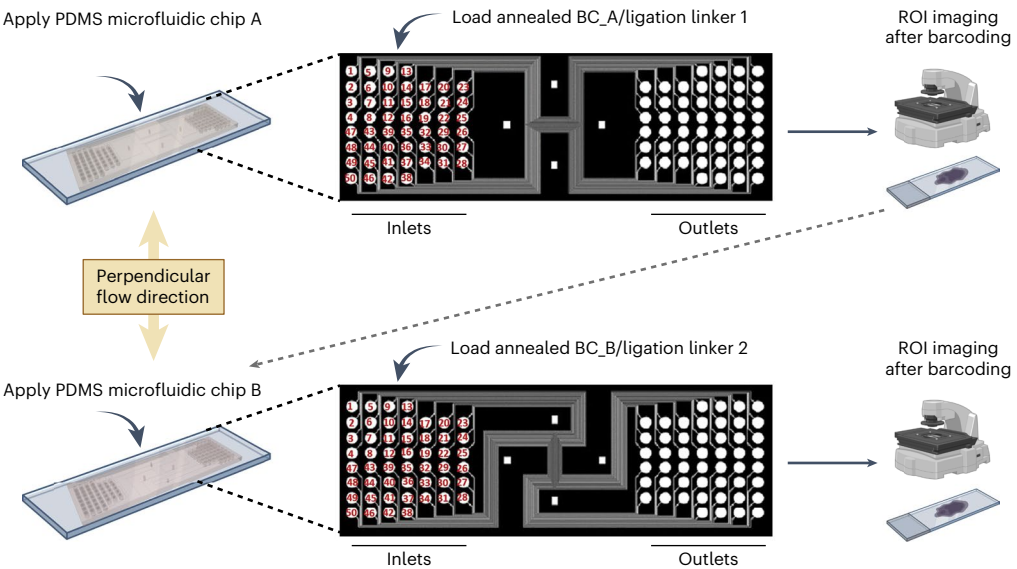

Fig. 4 | Workflow of deterministic barcoding in tissue. Apply the first PDMS microfluidic chip (chip A) onto the tissue section and load annealed BC_A/ligation linker 1 into each inlet. Image the section after in situ barcoding. Then, apply the second PDMS microfluidic chip (chip B), load annealed BC_B/ligation linker 2 and image the section after in situ barcoding. Patterns of a 50-channel × 50-channel chip, including the inlet loading order, are shown in the illustration. Of note, the chip pattern is symmetrical, so any one of the two sides can be used as the inlet side and the other as the outlet side.

73. Wash the tissue section:
    - Add 500 µL 1× NEB buffer 3.1 onto the section
    - Incubate at 20–25 °C for 5 min and carefully aspirate and discard the buffer
    - Dip the slide in diH_2O for 1 min and dry the slide with compressed air
74. Scan the tissue section with the EVOS FL auto imaging system.

Deterministic barcoding in tissue (barcode B)

● **TIMING 45–60 min**

▲ **CRITICAL** This procedure is required for both spatial-ATAC-RNA-seq and spatial-CUT&Tag-RNA-seq experiments.

75. Place PDMS microfluidic chip B onto the tissue ROI (Fig. 4).
    - Make sure the tissue section on the slide is fully dry before applying the PDMS chip
    - Clamp the PDMS chip and the slide tightly with the acrylic clamp described above
      ▲ **CRITICAL STEP** Channels of the PDMS microfluidic chip should be free of dust to ensure smooth flow of solution.
76. Prepare the barcode mixture containing in-situ ligation mix and annealed BC_B/ligation linker 2 as described in Steps 66–67.
77. Load 5 µL barcode mixture from each well of the 96-well plate to each inlet of the PDMS microfluidic chip, following the sequence of arrangement shown in Fig. 4.
    ▲ **CRITICAL STEP** Make sure the mixtures containing the uniquely index barcodes are added to the correct inlet positions since the barcodes correspond to the *y*-axis coordinates used in data analysis (Fig. 4).
78. Apply a vacuum cap on the outlet reservoir and let the barcode mixture slowly flow through the channels for 5 min.
    ▲ **CRITICAL STEP** Make sure all the channels over the tissue ROI are filled with the barcode mixture.
    ▲ **CRITICAL STEP** Start with a gentle air pressure and increase the pressure gradually. Never apply vacuum to the channels with high pressure to avoid leakage and tissue detachment.
79. Place the slide with the PDMS chip in a humidified chamber and incubate at 37 °C for 30 min.
80. Suck solution out of the inlets and outlets by applying a vacuum aspiration system.
81. Carefully release the acrylic clamp and remove the PDMS microfluidic chip.
82. Wash the tissue section:
    - Add 500 µL 1× PBS–RI (supplemented with an extra 2 µL SUPERase In RNase Inhibitor) onto the section
    - Incubate at 20–25 °C for 5 min and carefully aspirate and discard the liquid
    - Dip the slide in diH_2O for 1 min and dry the slide with compressed air
83. Scan the tissue section with the EVOS FL auto imaging system.

Reverse crosslinking

● **TIMING 150 min + overnight incubation**

▲ **CRITICAL** This procedure is required for both spatial-ATAC-RNA-seq and spatial-CUT&Tag-RNA-seq experiments.

84. Attach a small PDMS reservoir on the tissue slide so that the reservoir opening is directly over the barcoded tissue ROI.
85. Clamp the small PDMS reservoir and the slide tightly with the acrylic clamp.
86. Prepare the reverse crosslinking buffer (see 'Reagent setup') and add 100 µL into the PDMS reservoir.
87. Place the slide in a humidifying chamber and incubate at 55 °C for 2 h to reverse formaldehyde crosslinks.
88. After incubation, collect the tissue lysate:
    - Transfer ~90 µL lysate to a 1.5 mL tube
    - Add 50 µL reverse crosslinking buffer to wash the reservoir and pool with the lysate
    - Seal the tube with a Parafilm
89. Incubate the lysate at 55–65 °C with rotation overnight.

Streptavidin-bead affinity pulldown

● **TIMING 1–2 h**

▲ **CRITICAL** This procedure is required for both spatial-ATAC-RNA-seq and spatial-CUT&Tag-RNA-seq experiments. A video illustration for Steps 91–96 is available in Supplementary Video 4.

90. Purify the tissue lysate with the Zymo DNA Clean & Concentrator-5 according to the manufacturer's instructions.
    - Add 5 volumes of DNA binding buffer provided in the kit to each sample
    - Elute the tissue lysate with 150 µL nuclease-free H_2O

    ◆ **TROUBLESHOOTING**
91. Wash 40 µL Dynabeads MyOne Streptavidin C1 beads with 200–400 µL 1× B&W-T buffer three times.
92. Resuspend the beads with 150 µL 2× B&W buffer supplemented with 3 µL SUPERase In RNase Inhibitor.
93. Thoroughly mix the bead suspension with the purified tissue lysate from Step 90 by pipetting up and down in a 1.5 mL tube.
94. Place the sample on a lab rotator (see 'Equipment') and incubate at 20–25 °C for 1 h.
95. Place the tube on a magnetic stand and separate the supernatant from the beads. Transfer the supernatant into a new 1.5 mL tube.

    ▲ **CRITICAL STEP** The supernatant contains genomic fragments that will be used for spatial epigenomic library preparation. The beads contain cDNA and will be used for spatial transcriptomic library preparation.
96. Purify the supernatant (genomic fragments) with the Zymo DNA Clean & Concentrator-5:
    - Add 5 volumes of DNA binding buffer provided in the kit to each sample
    - Elute the sample with 20 µL nuclease-free H_2O

    ■ **PAUSE POINT** Store all bead samples and fragment elution samples at −80 °C.

Template switching for the spatial-RNA-seq library

● **TIMING 120–150 min**

▲ **CRITICAL** This procedure is required for both spatial-ATAC-RNA-seq and spatial-CUT&Tag-RNA-seq experiments.

97. Prepare the TSO mix (see 'Reagent setup').
98. Wash the beads from Step 95 twice with 400 µL of 1× B&W-T buffer.
99. Wash the beads once with 10 mM Tris pH 8.0 containing 0.1% (vol/vol) Tween-20.
100. Resuspend the beads with the 220 µL TSO mix.
101. Perform the template switching reaction:
    - Seal the tube with a Parafilm
    - Incubate at 20–25 °C for 30 min on a laboratory rotator (see 'Equipment')
    - Then incubate at 42 °C for 90 min, with gentle agitation (300 rpm) on a thermocycler (see 'Equipment')

    ◆ **TROUBLESHOOTING**

Spatial-RNA-seq library preparation

● **TIMING 3 h**

▲ **CRITICAL** This procedure is required for both spatial-ATAC-RNA-seq and spatial-CUT&Tag-RNA-seq experiments.

102. Wash the beads from Step 101 once with 400 µL of 10 mM Tris containing 0.1% (vol/vol) Tween-20 and once with nuclease-free H_2O.
    - Resuspend the beads with 110 µL of 2× Kapa HiFi HotStart Master Mix, 8.8 µL of 10 µM PCR primer 1, 8.8 µL of 10 µM PCR primer 2 and 92.4 µL of nuclease-free H_2O.
    - Split the 200 µL bead suspension into four PCR tubes with 50 µL in each.

103. Perform PCR with the following protocol:

Step	No. of cycles	Temperature	Time
1	1	95 °C	3 min
2	5	98 °C	20 s
		65 °C	45 s
		72 °C	3 min

104. Place the tubes on a magnetic stand and transfer the supernatant from each sample to a new PCR tube.
105. For each sample, add EvaGreen at a 1× concentration. Perform qPCR with the following protocol:

Step	No. of cycles	Temperature	Time
1	1	95 °C	3 min
2	15	98 °C	20 s
		65 °C	20 s
		72 °C	3 min
3	1	72 °C	5 min
4	1	4 °C	Hold

106. Watch the qPCR amplification in real time and stop the reaction (before the 15 cycles) once the qPCR signal begins to plateau.
▲ **CRITICAL STEP** Avoid overamplification of the cDNA. Failure in doing so may lead to reduced amplicon diversity in the library.
107. Purify the PCR product with 0.8× Ampure XP beads:
    - Prewarm the Ampure XP beads at 20–25 °C for 30 min before use for optimal performance
    - Freshly prepare 80% (vol/vol) ethanol
    - Combine the PCR products from all tubes
    - Add 0.8× Ampure XP beads to the combined PCR product. Mix thoroughly by pipetting or vortexing
    - Incubate the sample at 20–25 °C for 10 min
    - Place the sample on a magnet and incubate at 20–25 °C for 10 min until the liquid is clear
    - Carefully remove and discard the supernatant
    - While keeping the sample on the magnet, add 200 µL 80% ethanol
    - Incubate the sample on the magnet for 1 min
    - Carefully remove and discard the supernatant
    - Repeat the ethanol wash once
    - Briefly spin and carefully remove excess ethanol with a 20 µL pipette. Make sure the beads are not dried out
    - Remove the sample from the magnet and resuspend the beads with 10 µL of nuclease-free H_2O
    - Thoroughly mix the sample by pipetting or vortexing
    - Incubate the sample at 20–25 °C for 5–10 min to elute the DNA off the beads
    - Briefly spin down the tube and place it on the magnet until the solution becomes clear
    - Transfer the clear supernatant to a DNA LoBind microcentrifuge tube
    ■ **PAUSE POINT** The purified product can be stored at −20 °C for over 1 week.
108. Check the size distribution and concentration of the cDNA PCR product with a TapeStation instrument using the TapeStation High Sensitivity D5000 ScreenTape following the manufacturer's instruction.
109. Prepare sequencing library with the Nextera XT Library Prep kit:
    - Based on the concentration determined in the last step, take the volume needed to obtain 1–3 ng of purified cDNA product, and dilute in nuclease-free H_2O to a total of 5 µL

- Add 10 µL of tagment DNA buffer and 5 µL of amplicon tagment mix to bring the total volume to 20 µL
- Incubate at 55 °C for 5 min
- After incubation, add 5 µL NT buffer. Mix thoroughly by pipetting and incubate at 20–25 °C for 5 min

◆ **TROUBLESHOOTING**

110. Add the following reagents to the mix, in this order: 15 µL of PCR master mix, 1 µL of 10 µM i5 primer (for example, N501), 1 µL of 10 µM indexed i7 primer, 8 µL of nuclease-free H_2O. Mix thoroughly by pipetting.
111. Perform PCR on a thermal cycler with the following protocol:

Step	No. of cycles	Temperature	Time
1	1	95 °C	30 s
2	12	95 °C	10 s
		55 °C	30 s
		72 °C	30 s
3	1	72 °C	5 min
4	1	4 °C	Hold

112. Purify the PCR product with 0.7× Ampure XP beads:
    - Prewarm the Ampure XP beads at 20–25 °C for 30 min before use for optimal performance
    - Freshly prepare 80% (vol/vol) ethanol
    - Add 35 µL beads to the PCR product. Mix thoroughly by pipetting or vortexing.
    - Incubate the sample at 20–25 °C for 10 min
    - Place the sample on a magnet and incubate at 20–25 °C for 10 min until the liquid is clear
    - Carefully remove and discard the supernatant
    - While keeping the sample on the magnet, add 200 µL of 80% ethanol
    - Incubate the sample on the magnet for 1 min
    - Carefully remove and discard the supernatant
    - Repeat the ethanol wash once
    - Briefly spin, place the sample on the magnet and carefully remove excess ethanol with a 20 µL pipette. Process the beads for subsequent elution within 3 min to make sure the beads are not dried out
    - Remove the sample from the magnet and resuspend the beads with 20 µL of nuclease-free H_2O
    - Thoroughly mix the sample by pipetting or vortexing
    - Incubate the sample at 20–25 °C for 5–10 min to elute the DNA off the beads
    - Briefly spin down the tube and place it on the magnet until the solution becomes clear
    - Transfer the clear supernatant to a DNA LoBind microcentrifuge tube

    ■ **PAUSE POINT** The purified library can be stored at −20 °C for over 3 months.

Spatial-ATAC-seq library preparation

● **TIMING 1–2 h**

▲ **CRITICAL** This procedure is only required for the spatial-ATAC-RNA-seq assay.

113. Mix the 20 µL purified DNA genomic fragments from Step 96 (the supernatant from the bead separation) with 25 µL NEBNext High-Fidelity 2× PCR Master Mix, 2.5 µL 25 µM i5 primers (for example, N501) and 2.5 µL 25 µM indexed i7 primers.

    ▲ **CRITICAL STEP** Record the sequences of the i5/i7 indexes. Use differently indexed i5/i7 primer pairs for preparation of different libraries that will be pooled during sequencing.

114. Perform PCR pre-amplification on a thermal cycler with the following protocol:

Step	No. of cycles	Temperature	Time
1	1	72 °C	5 min
2	1	98 °C	30 s
3	5	98 °C	10 s
		63 °C	30 s
		72 °C	1 min
4	1	72 °C	1 min
5	1	4 °C	Hold

◆ **TROUBLESHOOTING**

115. Take 5 µL pre-amplified PCR products and create a qPCR reaction mix with the recipe below (total volume 15 µL). Place the remaining 45 µL PCR products on ice.
Place the remaining 45 µL PCR products on ice.

Reagent	Volume (µL)
Pre-amplified PCR products	5
NEBNext High-Fidelity 2× PCR Master Mix	5
Nuclease-free water	3.25
i5 primer (25 µM)	0.5
i7 primer (25 µM)	0.5
20× EvaGreen	0.75
Total	15

116. Perform qPCR with the following conditions to determine the appropriate number of PCR cycles to use in Step 117.

Step	No. of cycles	Temperature	Time
1	1	98 °C	30 s
2	20	98 °C	10 s
		63 °C	30 s
		72 °C	1 min

After the 20-cycle amplification is completed, determine the number of PCR cycles remaining for the library as the number of qPCR cycles required to reach 1/3 of saturation. For example, if the fluorescence intensity reaches 1,500 when amplification is saturated, look for the number of qPCR cycles at which an intensity of 500 can be reached.

117. Perform PCR on the remaining 45 µL products with the following conditions:

Step	No. of cycles	Temperature	Time
1	1	72 °C	5 min
2	1	98 °C	30 s
3	(Determined by qPCR in Step 116)	98 °C	10 s
		63 °C	30 s
		72 °C	1 min
4	1	72 °C	1 min
5	1	4 °C	Hold

118. Purify the PCR products with 1× Ampure XP beads (45 µL):
Prewarm the Ampure XP beads at 20–25 °C for 30 min before use for optimal performance
- Freshly prepare 80% (vol/vol) ethanol
- Add 45 µL beads to the PCR products. Mix thoroughly by pipetting or vortexing
- Incubate the sample at 20–25 °C for 10 min
- Place the sample on a magnet and incubate at 20–25 °C for 10 min until the liquid is clear

- Carefully remove and discard the supernatant
- While keeping the sample on the magnet, add 200 µL of 80% ethanol
- Incubate the sample on the magnet for 1 min
- Carefully remove and discard the supernatant
- Repeat the ethanol wash once
- Briefly spin the tube, place on the magnet and carefully remove excess ethanol with a 20 µL pipette and discard. Make sure the beads have not dried out
- Remove the sample from the magnet and resuspend the beads with 20 µL of nuclease-free H_2O
- Thoroughly mix the sample by pipetting or vortexing
- Incubate the sample at 20–25 °C for 5–10 min to elute the DNA off the beads
- Briefly spin and place the tube on the magnet until the solution becomes clear
- Transfer the clear supernatant to a DNA LoBind microcentrifuge tube

■ **PAUSE POINT** The purified library can be stored at −20 °C for over 3 months.

Spatial-CUT&Tag library preparation

● **TIMING 1–2 h**

▲ **CRITICAL** This procedure is only required for the spatial-CUT&Tag-RNA-seq assay. Though we present using qPCR as an approach to determine the number of PCR cycles needed for spatial-ATAC-seq library amplification in the above section, this approach is optional for spatial-CUT&Tag libraries. Nevertheless, we still recommend performing qPCR in a similar manner to estimate the required PCR cycle number if libraries are generated from samples of different sample types and different section thicknesses.

119. Mix the 20 µL purified genomic DNA fragments from Step 96 (the supernatant from the bead separation) with 25 µL NEBNext High-Fidelity 2× PCR Master Mix, 2.5 µL 10 µM i5 primers (for example, N501) and 2.5 µL 10 µM indexed i7 primers.

▲ **CRITICAL STEP** Record the sequences of the i5/i7 indexes. Use differently indexed i5/i7 primer pairs for preparation of different libraries that will be pooled during sequencing.

◆ **TROUBLESHOOTING**

120. Perform PCR on a thermal cycler with the following protocol:

Step	No. of cycles	Temperature	Time
1	1	58 °C	5 min
2	1	72 °C	5 min
3	1	98 °C	30 s
4	12	98 °C	10 s
		60 °C	10 s
		72 °C	1 min
5	1	72 °C	1 min
6	1	4 °C	Hold

121. Purify the PCR products with 1.3× Ampure XP beads (65 µL):
    - Prewarm the Ampure XP beads at 20–25 °C for 30 min before use for optimal performance
    - Freshly prepare 80% (vol/vol) ethanol
    - Add 65 µL beads to the PCR products. Mix thoroughly by pipetting or vortexing
    - Incubate the sample at 20–25 °C for 10 min
    - Place the sample on a magnet and incubate at 20–25 °C for 10 min until the liquid is clear
    - Carefully remove and discard the supernatant
    - While keeping the sample on the magnet, add 200 µL of 80% ethanol
    - Incubate the sample on the magnet for 1 min
    - Carefully remove and discard the supernatant
    - Repeat the ethanol wash once
    - Briefly spin the tube, place it on the magnet and carefully remove excess ethanol with a 20 µL pipette and discard. Make sure the beads have not dried out

- Remove the sample from the magnet and resuspend the beads with 20 µL of nuclease-free H_2O
- Thoroughly mix the sample by pipetting or vortexing
- Incubate the sample at 20–25 °C for 5–10 min to elute the DNA off the beads
- Briefly spin and place the tube on the magnet until the solution becomes clear
- Transfer the clear supernatant to a DNA LoBind microcentrifuge tube

■ **PAUSE POINT** The purified library can be stored at −20 °C and for over 3 months.

Library visualization and next-generation sequencing

● **TIMING variable**

122. Visualize all RNA-seq, ATAC-seq and CUT&Tag libraries with a TapeStation instrument using the TapeStation High Sensitivity D5000 ScreenTape following the manufacturer's instruction.

◆ **TROUBLESHOOTING**

123. Sequence libraries. All RNA-seq, ATAC-seq and CUT&Tag libraries, generated with the provided i5/i7 primers, can be sequenced on an Illumina NovaSeq 6000 paired-end 150 bp platform.
    - Libraries of different modalities, or libraries generated from different samples, can be pooled for sequencing if they are indexed with different pairs of i5/i7 sequences
    - For all libraries, we typically target a sequencing depth of at least 40,000 reads per pixel

    ▲ **CRITICAL STEP** To ensure optimal sequencing performance, please contact the sequencing service center for a recommended final volume and concentration of the library.

Sequencing data processing

● **TIMING variable**

124. Process and analyze Fastq sequencing data of a spatially resolved transcriptomics library with previously described pipelines (https://github.com/rongfan8/DBiT-seq)[15].
125. Process and analyze Fastq sequencing data of a spatially resolved epigenomics library (spatial-ATAC-seq or spatial-CUT&Tag) with previously described pipelines (https://github.com/dyxmvp/Spatial_ATAC-seq and https://github.com/dyxmvp/spatial-CUT-Tag)[23,24].
126. Each pixel is identified by a unique 16 bp barcode sequence (8 bp barcode A as the *y*-axis coordinate and 8 bp barcode B as the *x*-axis coordinate) and has both transcriptomic and epigenomic sequencing profiles, which can be analyzed separately or jointly with existing multimodal integration methods[61,62].

Troubleshooting

Troubleshooting advice can be found in Table 1.

Table 1 | Troubleshooting advice

Step	Problem	Possible reason	Solution
6	Tissue section falling off from the slide	The section is not fully dried before reagent treatment	At the end of Step 3, after warming up, fully dry the tissue with cool compressed air
122 and 124	Compromised transcriptomics library quality (<200 genes or <500 UMIs per pixel)	RNA loss during experiment	Reduce the duration of the tissue section's exposure to water at Step 8 and Step 22. For example, instead of dipping the slide into water for 1 min, use a pipette to gently flow water over the tissue section for ~10 s. Do not expose the whole slide to water for over 1 min, otherwise the RNA molecules that have not been crosslinked may dissolve in the water
			At Step 63, perform post-RT fixation with 4% (wt/vol) formaldehyde for 5 min at 20–25 °C, which can prevent cDNA being washed away during deterministic barcoding

Table 1 (continued) | Troubleshooting advice

Step	Problem	Possible reason	Solution
122 and 124	Compromised transcriptomics library quality (<200 genes or <500 UMIs per pixel) in spatial-ATAC-RNA-seq	Insufficient RNA capture	At Step 13 of permeabilization, double the concentration of NP-40, Tween 20 and digitonin. This will improve cDNA capture efficiency while not significantly affecting chromatin transposition
38	Inappropriate antibody dilution ratio	For antibodies not validated by this protocol, the optimal concentration may vary depending on the specificity of the antibody, species and/or type of sample used	Titrate antibody concentration in a range from 1:10 to 1:100. We recommend to try different antibodies showing optimal performance in immunofluorescence staining applications
90	Reduced library yield during reverse crosslinking	For the RNA–cDNA hybrid complex, RNA degradation occurs during overnight incubation and small fragments and single-strand cDNA may not be fully recovered	Six volumes of DNA binding buffer should be used. Incubate at 20–25 °C for at least 2 min before centrifugation
			Eluting the tissue lysate with water that has been warmed up to 85 °C may also prevent material loss
101	Reduced library yield during template switching	Disrupted structure of RNA–cDNA hybrid complex	Prevent RNA degradation. Make sure the TSO mix contains RNase inhibitors and the mix is prepared fresh and placed on ice immediately
109 and 122	Presence of long fragments (>700 bp) in the transcriptomics library and low concentration of fragments between 300 and 700 bp, suggesting compromised tagmentation in spatial-RNA-seq library preparation	Too much total cDNA product is used	Do not use more than 3 ng cDNA for Nextera library preparation to avoid insufficient tagmentation
113 and 119	Reduced yield of spatial epigenomic library	Inappropriate preparation of PCR master mix	We do not recommend replacing NEBNext High-Fidelity 2× PCR Master Mix with other forms of PCR master mix, since NEBNext High-Fidelity 2× PCR Master Mix is a non-hot-start PCR master mix efficient in filling gaps generated in tagmentation[49]
122	Low concentration of the spatial epigenomic library	Insufficient Tn5 or pA–Tn5 transposition	For spatial-ATAC-seq, the incubation time at Step 28 may be increased to 45–60 min. The dilution factor of loaded Tn5 in the ATAC transposition mix preparation (now 1:20) may be changed to 1:10–20
			For spatial-CUT&Tag, the dilution factor of pA–Tn5 at Step 46 (now 1:100) may be changed to 1:50–100
			For both Tn5 and pA–Tn5 transposition, pipetting up and down 10 times every 15 min during the reaction may also increase the transposition efficiency

Timing

Spatial-ATAC-RNA-seq

Day 1

Tn5 transposome assembly (Box 1), 1–2 h
Spatial barcode annealing (Box 2), 2 h
Preparation of PDMS associated equipment (Box 3), 3–4 h
Reagent setup

Day 2

Steps 1–6: tissue fixation, 30–45 min
Steps 7–19: tissue permeabilization, 30–45 min
Steps 27–37: chromatin transposition, 60–75 min
Steps 56–63: RT, 2–2.5 h
Steps 64–74: deterministic barcoding in tissue (barcode A), 45–60 min
Steps 75–83: deterministic barcoding in tissue (barcode B), 45–60 min
Steps 84–89: reverse crosslinking, 2.5 h (followed by overnight incubation)

Days 3–4
Steps 90–96: streptavidin bead affinity pulldown, 1–2 h
Steps 97–101: template switching for the spatial-RNA-seq library, 2–2.5 h
Steps 102–112: spatial-RNA-seq library preparation, 3 h
Steps 113–118: spatial-ATAC-seq library preparation, 1–2 h
Step 122: library visualization, 20–30 min

Spatial-CUT&Tag-RNA-seq
Day 1
pA–Tn5 transposome assembly (Box 1), 2 h
Spatial barcoding annealing (Box 2), 2 h
Preparation of PDMS associated equipment (Box 3), 3–4 h
Reagent setup

Day 2
Steps 1–6: tissue fixation, 30–45 min
Steps 20–26: tissue permeabilization, 10–20 min
Steps 38–39: antibody binding and tagmentation, overnight

Day 3
Steps 40–55: antibody binding and tagmentation, 3–4 h
Steps 56–63: RT, 2–2.5 h
Steps 64–74: deterministic barcoding in tissue (barcode A), 45–60 min
Steps 75–83: deterministic barcoding in tissue (barcode B), 45–60 min
Steps 84–89: reverse crosslinking, 2.5 h (followed by overnight incubation)

Day 4-5
Steps 90–96: streptavidin bead affinity pulldown, 1–2 h
Steps 97–101: template switching for the spatial-RNA-seq library, 2–2.5 h
Steps 102–112: spatial-RNA-seq library preparation, 3 h
Steps 119–121: spatial-CUT&Tag library preparation, 1–2 h
Step 122: library visualization, 20–30 min

Anticipated results

Library visualization

The concentration and fragment distribution of all libraries should be examined with a TapeStation or BioAnalyzer electropherogram instrument before next-generation sequencing. For the spatial-RNA-seq library in both spatial-ATAC-RNA-seq and spatial-CUT&Tag-RNA-seq, we first visualize the cDNA library after template switching and PCR amplification (Step 108). The cDNA library is expected to have an average length of 600–1,000 bp (Fig. 5a). The concentration of a cDNA library may exceed 10 ng/μL, in which case only a proportion of cDNA products is used for subsequent library preparation with the Nextera XT Library Prep kit, while the remainder of the cDNA sample can be stored for potential troubleshooting or replicate preparation. The Nextera library preparation procedures involve cDNA tagmentation and the final library should have a lower average fragment length, typically in a range of 300–700 bp (Fig. 5b) with a concentration over 10 ng/μL.

Multiple peaks are expected to appear in the trace of a spatial-ATAC-seq library, reflecting the periodicity of the chromatin structure, and corresponding to nucleosome-free, mono-nucleosome, di-nucleosome or multinucleated fragments (Fig. 5c). The first nucleosome-free peak should have an average insert size of 300–330 bp and usually exhibits higher density than the nucleosome peaks. In some cases, the mono-nucleosome peak may show a stronger signal than the nucleosome-free peak and this should not significantly impact the library quality

(Extended Data Fig. 1). The concentration of a spatial-ATAC-seq library (between 200 and 5,000 bp) is typically over 10 ng/μL, though a library with a concentration between 5 and 10 ng/μL may also be acceptable. We have recently described the expected library visualization

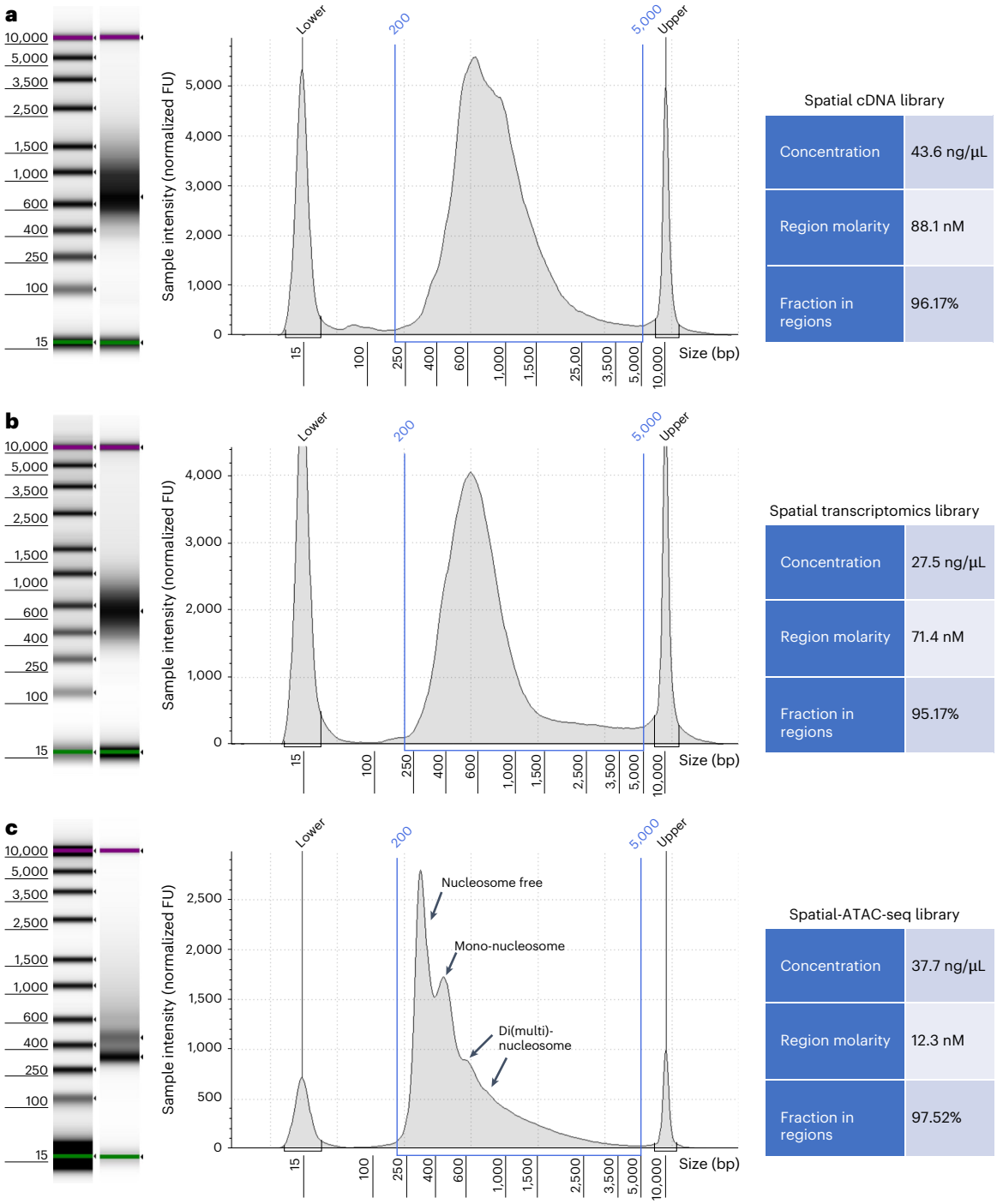

Fig. 5 | Anticipated results of library visualization. a, A TapeStation D5000 electropherogram showing length distribution of a cDNA sample of a spatial transcriptomics library generated from a human brain sample. The cDNA sample is then used for final library preparation with the Nextera XT Library Prep kit. **b**, A TapeStation D5000 electropherogram showing length distribution of a spatial transcriptomics library generated after Nextera library preparation on the sample shown in Fig. 5a. **c**, TapeStation D5000 electropherogram (High-Sensitivity) showing fragment distribution of a spatial-ATAC-seq library, with the expected chromatin structure periodicity indicated. The tables on the right indicate the concentration, molarity and fractions of fragments in the select region (200–5,000 bp) for each library. FU, fluorescence units.

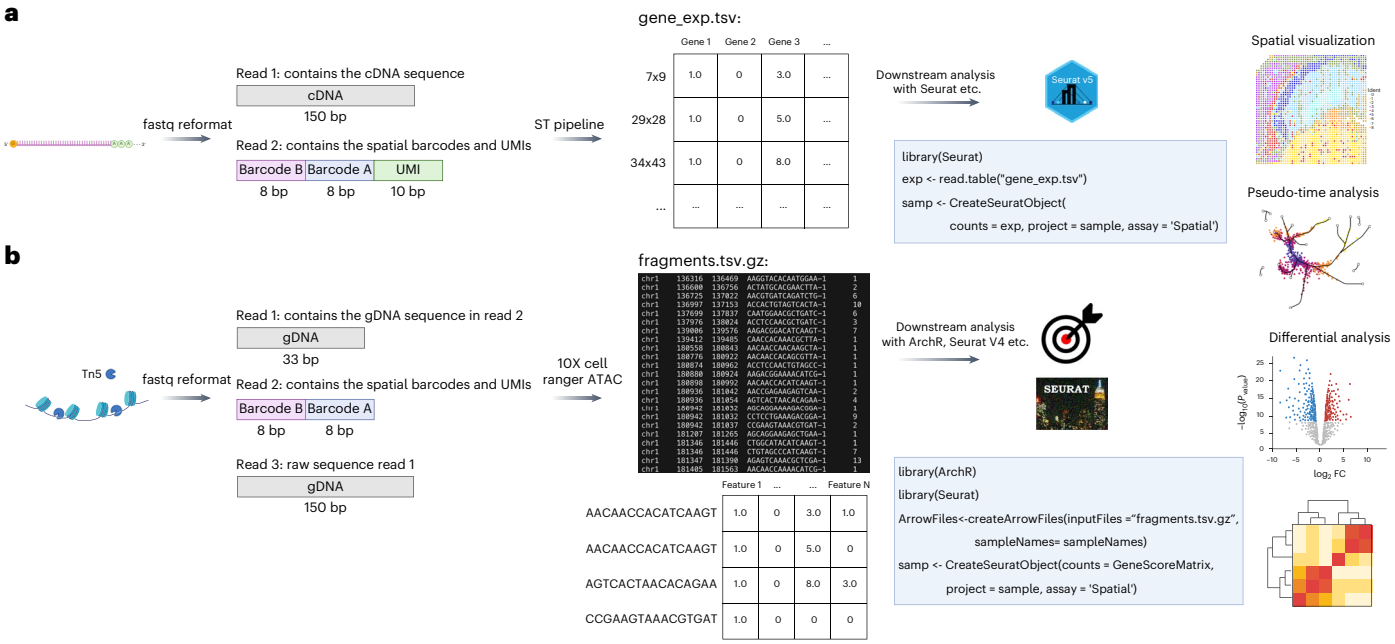

Fig. 6 | Bioinformatics pipelines for analyzing spatially resolved epigenome-transcriptome data. a, For the transcriptomics data, raw sequencing data in fastq format are filtered and reformatted so that read 1 and read 2 represent cDNA and spatial barcodes, respectively. The ST Pipeline[57] is then used for reference genome mapping, gene counting and annotations, while the spatial coordinates are translated from a format of 16 bp sequences to a format of $x \times y$ coordinates. The annotated count matrix can be imported into various downstream pipelines such as Seurat[7] in R or Scanpy[64] in Python. **b**, For the epigenomics data (ATAC-seq or CUT&Tag), raw sequencing data in fastq format are filtered and reformatted so that read 1 and read 3 represent paired-end gDNA and read 2 represents spatial barcodes. The filtered and reformatted data can be then processed with 10X Cell Ranger ATAC. This generates a fragment file in the bed format, which can be used for various downstream pipelines such as ArchR[59] and Seurat[7] in R.

results of a spatial-CUT&Tag library[25] and we anticipate a similar result for the spatial-CUT&Tag-RNA-seq library on histone modifications. The trace may also indicate the chromatin structure periodicity, although mono-nucleosome-sized fragments are often predominantly enriched.

Data quality control and analysis

A workflow of bioinformatic pipelines for processing and analyzing the data is presented in Fig. 6. For the spatial-ATAC-RNA-seq library, we typically obtain an average of 10,000–20,000 fragments per pixel with the fraction of fragments located in TSS regions as 15–25% (Fig. 7a). An average UMI number of 2,000–5,000 and gene number of 1,000–2,000 per pixel is expected (Fig. 7a). Libraries generated from human brain samples typically exhibit lower gene detection sensitivity and genome coverage than those generated from mouse brains, largely due to RNA degradation during human tissue dissection and storage.

For the spatial-CUT&Tag-RNA-seq library, a strong enrichment of fragments in TSS regions and in the called peaks should be expected for libraries targeting the H3K4me3 modification, which indicates active promoters (Fig. 7b). The H3K27me3 and H3K27ac modifications can indicate nonpromoter regions and therefore exhibit a lower fraction of fragments in TSSs. We typically obtain a similar range of UMI and gene numbers per pixel as those in spatial-ATAC-RNA-seq. Acceptable libraries are expected to have a minimum of 600 genes and 1,500 UMIs detected per pixel. Since the H3K27me3 modification usually marks repressive loci and H3K4me3/H3K27ac modifications are associated with gene activation, a routine analysis we perform on a spatial-CUT&Tag-RNA-seq dataset is to define a chromatin silencing score (CSS) with the H3K27me3 profile, as well as a gene activity score (GAS) with the H3K4me3 or H3K27ac profile[26]. The correlation of CSS or GAS with gene expression profiles can be analyzed (Fig. 7c) and, as expected, we typically observe a negative correlation of gene expression with CSS, as well as a positive correlation with GAS for most genes.

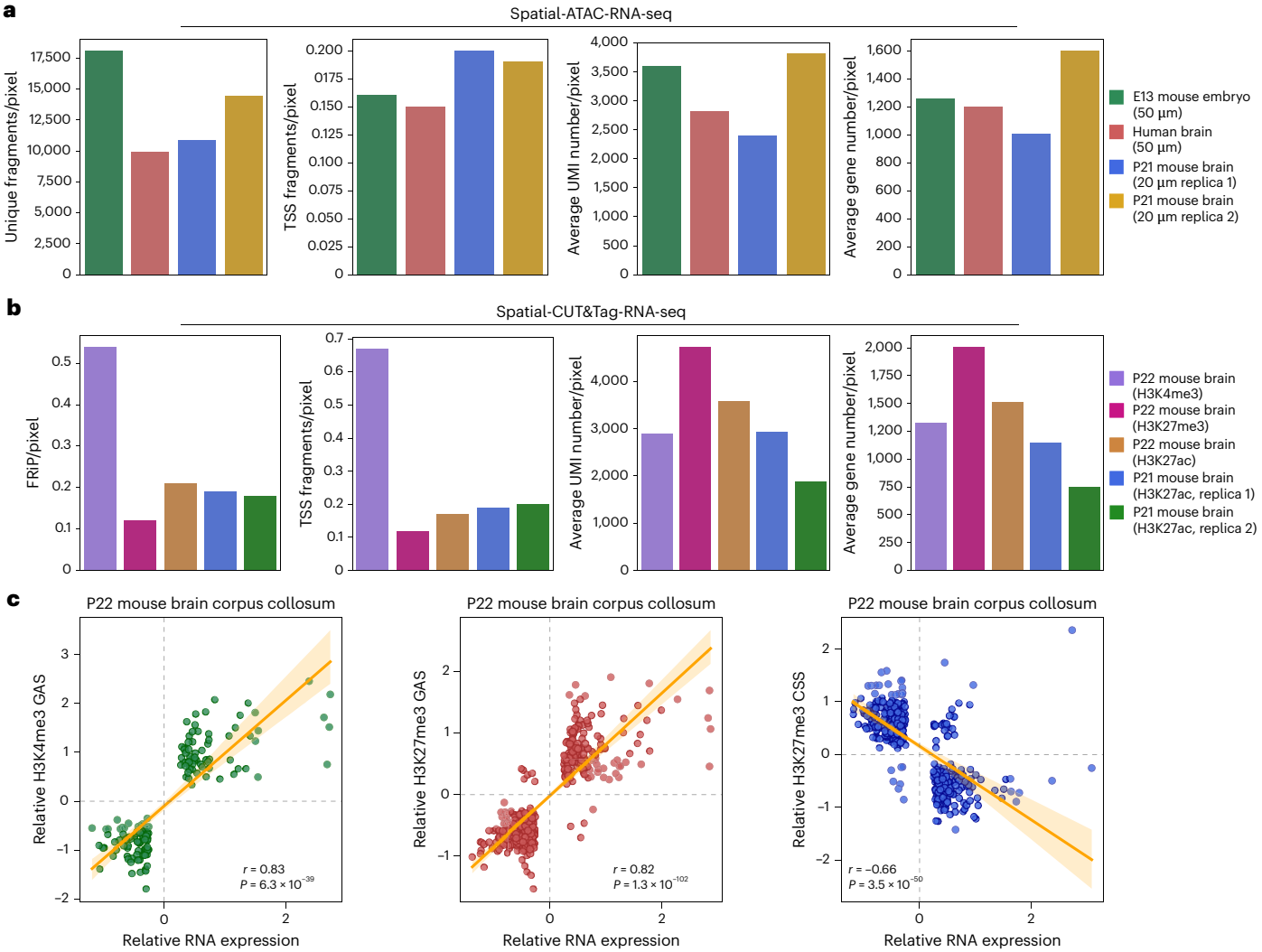

Fig. 7 | Spatial-ATAC-RNA-seq and spatial-CUT&Tag-RNA-seq data quality control. a, Unique fragments, fraction of fragments in TSSs, number of UMI and number of genes per pixel in spatial-ATAC-RNA-seq libraries. Sample names are indicated on the right with the microfluidic chip type specified in parenthesis. **b**, The fraction of reads in peaks (FRiP), fraction of fragments in TSSs, number of UMI and number of genes per pixel in spatial-CUT&Tag-RNA-seq libraries. Sample names and the profiled histone modifications are indicated on the right. All libraries are generated with microfluidic chips of 20 μm pixel size. **c**, Correlation plots of gene expression with H3K4me3 GAS, H3K27ac GAS and H3K27me3 CSS levels, respectively. The corpus callosum cluster of P22 mouse brain libraries is used for analysis. Pearson correlation coefficients (*r*) and *P* values are shown, with the confidence interval for the regression estimate set as 95. Original data and information about the samples are available in Zhang et al.[26]. In brief, the E13 mouse embryo sample was collected from one wild-type C57BL/6 mouse and sagittal frozen sections were prepared; the human brain sample was obtained from a 31-year-old Caucasian male donor; the mouse P21/P22 samples were obtained from the *Sox10:Cre-RCE:LoxP* line with a mixed C57BL/6×CD1 genetic background.

Before proceeding to downstream analysis such as dimensional reduction and differential marker identification, we encourage researchers to carefully evaluate the read mapping efficiency and remove observations (that is, pixels) with low quality. For example, we anticipate that over 80% of the filtered reads of a spatial transcriptomics library can be uniquely mapped with the corresponding reference genome during genome alignment, with a minimal fraction of reads that are mapped to multiple genomic loci or mismatched (Fig. 8a). For the spatial-ATAC-seq data, analyzing the distribution of fragment lengths (Fig. 8b) should reveal that the majority of fragments are between 30 and 147 bp in length, indicating open chromatin at nucleosome-free regions. Another small peak referring to the mono-nucleosome regions may also be identified at a range of 147–200 bp. The other larger fragments reflect di-nucleosome

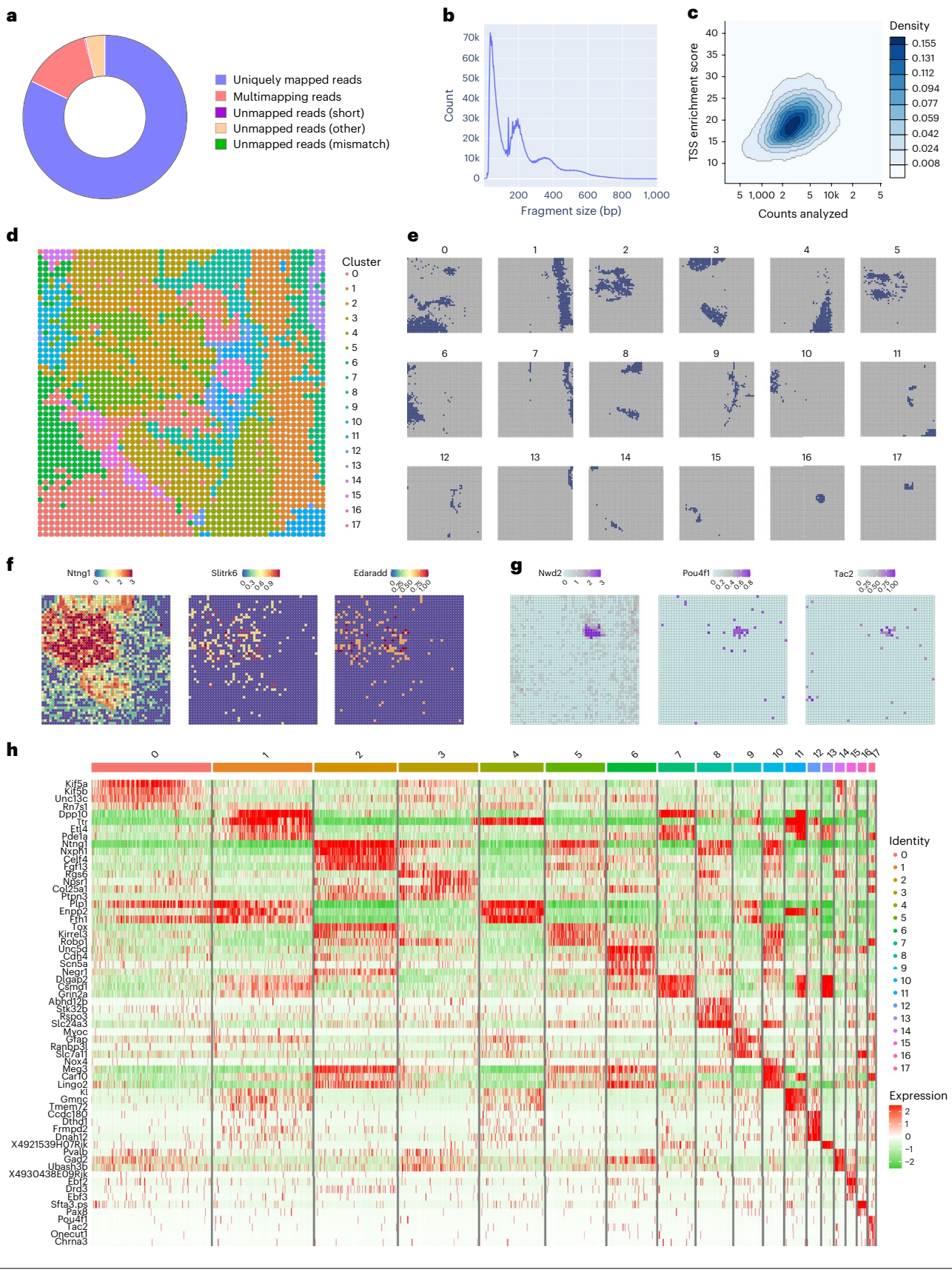
a
Uniquely mapped reads
Multimapping reads
Unmapped reads (short)
Unmapped reads (other)
Unmapped reads (mismatch)
b
Count
Fragment size (bp)
c
TSS enrichment score
Counts analyzed
Density
d
Cluster
e
f
Ntng1
Slitrk6
Edaradd
g
Nwd2
Pou4f1
Tac2
h
Identity
Expression

Fig. 8 | Evaluation and analysis of a spatial-ATAC-RNA-seq dataset. a, A pie chart showing the fraction of spatial-RNA-seq reads of different categories that are mapped with the reference genome. **b**, Length distribution of fragments obtained in a spatial-ATAC-seq data. **c**, Correlation of TSS enrichment score, calculated by snapATAC2 (ref. 60), with spatial-ATAC-seq fragments. Density indicates the number of observations. **d**,**e**, Spatial projection of 18 clusters together (**d**) or individually (**e**) on a mouse brain tissue, from a spatial-ATAC-RNA-seq data profiled with the 50-channel × 50-channel chips. **f**,**g**, Spatial feature plots for gene expression of *Ntng1*, *Slitrk6* and *Edaradd* (thalamus markers) (**f**) and *Nwd2*, *Pou4f1* and *Tac2* (habenula markers) (**g**). **h**, A heat map showing differentially expressed genes of each cluster, calculated in differential analysis with the Seurat FindMarker function[7]. Each row indicates a gene and each column indicates one of the 2,500 pixels. All data are derived from the same spatial-ATAC-RNA-seq dataset, which was generated from brain samples of one wild-type C57BL/6 12-week-old male mouse.

and multinucleosome signals and should account for a minimal fraction of the total fragments. For a spatial-ATAC-seq library and spatial-CUT&Tag library that targets promoter-associated antigens, it is crucial to examine the TSS enrichment level for the fragments (Fig. 8c) since they typically indicate open chromatin at promoter regions. A TSS enrichment score can be calculated by several publicly available tools (for example, Signac[58], SnapATAC2 (ref. 60)), and observations (that is, pixels) with lower TSS enrichment score may be removed in downstream analysis.

After dimensional reduction, Louvain or Leiden clustering analysis can be performed to group pixels into different clusters. The clusters can be visualized with their spatial information (that is, x and y coordinates) preserved (Fig. 8d) and we anticipate that the clustering pattern should be highly correlated with the anatomic distribution of different cell types. A simple approach that facilitates this analysis is to examine the spatial distribution of each cluster individually (Fig. 8e). In an experiment performed on the adult mouse brain tissue profiled at a resolution of 20 µm, as shown in Fig. 8d–h, for example, cluster 2 mostly indicates the brain thalamus and cluster 17 clearly marks the habenula region. Examining well-known marker genes of these anatomic regions[63], such as *Ntng1*, *Slitrk6* and *Edaradd* for the thalamus (Fig. 8f) and *Nwd2*, *Pou4f1* and *Tac2* for the habenula (Fig. 8g), confirms their region-specific gene expression pattern. In addition, differential analysis can be performed on distinct clusters[7] (Fig. 8h), in which the spatial transcriptomics data is often leveraged for cell type annotations.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Original data that are used to generate metrics plots in Figs. 7 and 8 are available in Source Data. Raw data for the illustrative results shown in this protocol are available in Zhang et al.[26]. Source data are provided with this paper.

Code availability

Codes for processing data of the associated publication[26] are available at https://github.com/di-0579/Spatial_epigenome-transcriptome_co-sequencing.

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Author contributions

H.L., N.F. and R.F. conceived, coordinated and designed the study. H.L., S.B., X.Q. and B.T. wrote the manuscript. H.L., S.B., A.A.F., D.Z., Z.B. and B.T. analyzed data and created figures. R.F. supervised the project and revised the manuscript. All authors read and approved the final manuscript.

Competing interests

R.F. is scientific founder of and advisor to IsoPlexis, Singleron Biotechnologies and AtlasXomics. The Yale University Provost's Office reviewed and managed the interests of R.F. in accordance with the University's conflict of interest policies. The other authors declare no competing interests.

Additional information

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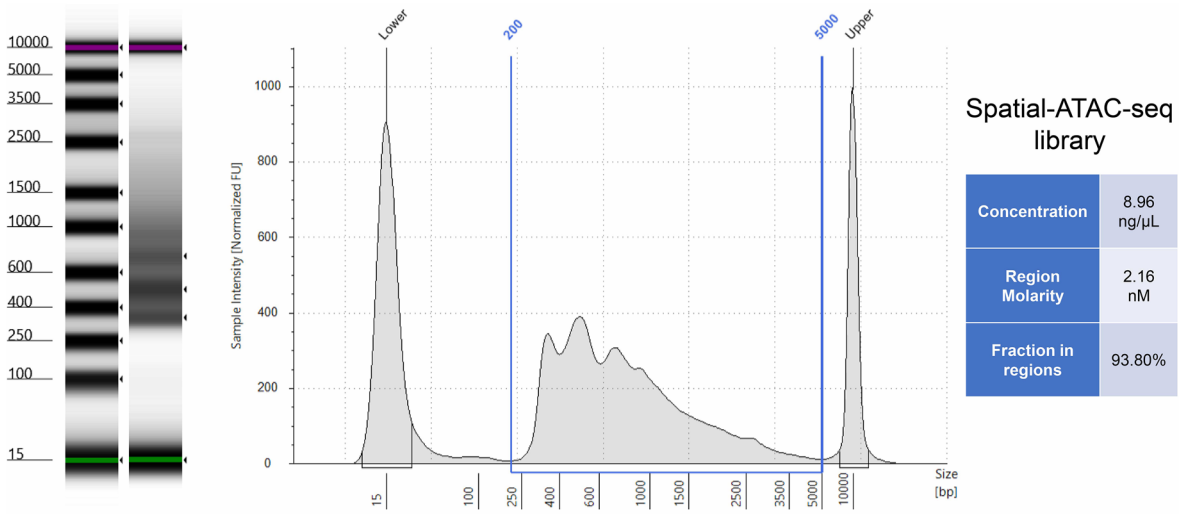

Extended Data Fig. 1 | Anticipated results of spatial-ATAC-seq library visualization. TapeStation D5000 electropherogram (High-Sensitivity) showing fragment distribution of a spatial-ATAC-seq library generated from a human brain sample. The table on the right panel indicates the concentration, molarity and fractions of fragments in the select region (200-5,000 bp).

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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	No software used for data collection
Data analysis	Cell Ranger ATAC v.1.2 (10XGenomics), ST pipeline v.1.7.2, Seurat v4.1, Signac v1.8 and SnapATAC2 v2.6.0 were used in data analysis.

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☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used

- α-H3K27me3 antibody (Cell Signaling Technology, cat no. 9733, https://scicrunch.org/resolver/AB_2616029); (http://antibodyregistry.org/AB_2616029)
- α-H3K4me3 antibody (Active Motif, cat no. 39159, https://scicrunch.org/resolver/AB_2615077); (http://antibodyregistry.org/AB_2615077)
- α-H3K27ac antibody (Abcam, cat no. ab177178, https://scicrunch.org/resolver/AB_2828007); (http://antibodyregistry.org/AB_2828007)
- Guinea Pig anti-Rabbit IgG Secondary antibody (Antibodies-Online, cat no. ABIN101961, https://scicrunch.org/resolver/AB_10775589); (http://antibodyregistry.org/AB_10775589)

Validation	• α-H3K27me3 antibody (Cell Signaling Technology, cat no. 9733, https://scicrunch.org/resolver/AB_2616029); Citations: Janssens DH, et al. (2024) Scalable single-cell profiling of chromatin modifications with sciCUT&Tag. Nature protocols, 19(1), 83. (PMID:37935964) DuCote TJ, et al. (2024) EZH2 Inhibition Promotes Tumor Immunogenicity in Lung Squamous Cell Carcinomas. Cancer research communications, 4(2), 388. (PMID:38265267) Refer to the manufacturer's description: https://www.cellsignal.com/products/primary-antibodies/tri-methyl-histone-h3-lys27-c36b11-rabbit-mab/9733?srsltid=AfmBOoq9evQvsxdKtW4qFIyg3SHuyxRRpYUq9MASpLB_BuKSjPegazPS • α-H3K4me3 antibody (Active Motif, cat no. 39159, https://scicrunch.org/resolver/AB_2615077); (http://antibodyregistry.org/AB_2615077) Citations: Janssens DH, et al. (2024) Scalable single-cell profiling of chromatin modifications with sciCUT&Tag. Nature protocols, 19(1), 83. (PMID:37935964) Del Vecchio A, et al. (2024) PCGF6 controls murine Tuft cell differentiation via H3K9me2 modification independently of Polycomb repression. Developmental cell, 59(3), 368. (PMID:38228142) Refer to the manufacturer's description: https://www.activemotif.com/catalog/details/39159 • α-H3K27ac antibody (Abcam, cat no. ab177178, https://scicrunch.org/resolver/AB_2828007); (http://antibodyregistry.org/AB_2828007) Citations: Bárcenas-Walls JR, et al. (2024) Nano-CUT&Tag for multimodal chromatin profiling at single-cell resolution. Nature protocols, 19(3), 791. (PMID:38129675) Wang C, et al. (2024) Serine synthesis sustains macrophage IL-1β production via NAD+-dependent protein acetylation. Molecular cell, 84(4), 744. (PMID:38266638) Refer to the manufacturer's description: https://www.abcam.com/en-us/products/primary-antibodies/histone-h3-acetyl-k27-antibody-ep16602-chip-grade-ab177178 • Guinea Pig anti-Rabbit IgG Secondary antibody (Antibodies-Online, cat no. ABIN101961, https://scicrunch.org/resolver/AB_10775589); (http://antibodyregistry.org/AB_10775589) Citations: Barisic D, et al. (2024) ARID1A orchestrates SWI/SNF-mediated sequential binding of transcription factors with ARID1A loss driving pre-memory B cell fate and lymphomagenesis. Cancer cell. (PMID:38458187) Janssens DH, et al. (2024) Scalable single-cell profiling of chromatin modifications with sciCUT&Tag. Nature protocols, 19(1), 83. (PMID:37935964) Refer to the manufacturer's description: https://www.antibodies-online.com/secondary-antibodies/101961/Guinea+Pig+anti-Rabbit+IgG+Heavy++Light+Chain+antibody+-+Preadsorbed/

Animals and other research organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research, and Sex and Gender in Research

Laboratory animals	Wild-type C57BL/6 12-week-old mouse
Wild animals	Not relevant to this protocol article.
Reporting on sex	Sex was not considered in the study design. Animal sex, if known, is indicated in the manuscript text.
Field-collected samples	Not relevant to this protocol article.
Ethics oversight	Animal materials were purchased from Zyagen. The in-house Institutional Animal Care and Use Committee of Zyagen review and approve all protocols.

Note that full information on the approval of the study protocol must also be provided in the manuscript.