1. **FFPE sections treatment**

Solution:

Graded series of ethanol solutions (Aladdin, Cat # E130059): 100%, 100%, 90%, 70%, 50%, 30% and Ultrapure Water;

Wash buffer: 1X PBS with 1U/μL Murine RNase inhibitor (Vazyme, Cat # R301);

2X SSC buffer: 2X SSC buffer with 1U/μL Murine RNase inhibitor.

FFPE samples of mouse tissues were prepared by HaoKe Biotechnology Co. Ltd. FFPE sections were cut from the paraffin block and were kept on at 60℃ for 2h. The sections were then washed twice with 5 ml Xylene (Aladdin, Cat # X112050) for 20 min at room temperature to deparaffinize. The sections were rehydrated with immersing in a graded series of ethanol solutions (Aladdin, Cat # E130059) from pure 100% to 30% for 2 min each time. The sections were washed three times with 4 ml pre-cold wash buffer at room temperature for 5 min.

The FFPE sections placed on the chip were decrosslinking using 0.1% pepsin (Sigma, Cat # P7000) in 0.01M HCl , incubated at 37℃ for 12 min and then washed three times with 2X SSC buffer.

2. **DNA block**

100 μL DNA polymerase mix:

44 μL dd$H_2O$,

10 μL 5X DNA polymerization buffer (M20 Genomics, Cat # R20123124),

20 μL 10 mM dNTP (NEB, Cat # N0447L),

12 μL 10 μM block primer (Supplementary table 4),

5 μL Murine RNase Inhibitor,

5 μL 10% Triton X 100,

4 μL DNA polymerase (M20 Genomics, Cat # R20123124)

The FFPE sections were incubated with the DNA polymerase mix at 37℃ for 30 min using a thermocycler adapter (10x Genomics). After incubation, the sections were washed with PBST (1X PBS with 0.05% T-ween 20) three times to wash away the residual blocking primers and primer dimers.

3. **Reverse transcription**

100 μL reverse transcription mix:

32 μL dd$H_2O$,

20 μL 5X reverse transcription buffer (M20 Genomics, Cat # R20114124),

19 μL 40% PEG8000 (Solarbio Cat # P8260),

5 μL 10% TritonX 100,

5 μL 10 mM dNTP,

10 μL 10 μM random primer (10 μM mix of TruRd2S-7N and TruRd2S-15T, Supplementary table 4),

5 μL Murine RNase Inhibitor,

5 μL reverse transcriptase (M20 Genomics, Cat#R20114124).

The FFPE sections were incubated with the reverse transcription mix with multiple annealing ramping from 8℃ to 42℃ and overnight at 42℃. After incubation, the sections were washed with 2X SSCT (2X SSC with 0.05% T-ween 20) three times to wash away the residual random primers and primer dimers.

4. **dA tailing**

100 μL dA tailing mix:

78 μL dd$H_2O$,

10 μL 10X TDT buffer (NEB, Cat # M0315S),

10 μL $CoCl_2$ buffer (NEB, Cat # M0315S),

1 μL 100mM dATP (Solarbio, Cat # R0141),

1 μL TdT enzyme (NEB, Cat # M0315S).

The FFPE sections were incubated with the dA tailing mix at 37℃

for 30 min and then were washed with 2X SSCT (2X SSC with 0.05% T-ween 20) three times.

5. **cDNA transfer**

50 μL transfer mix:

34.5 μL dd$H_2O$,

5 μL RNase H reaction buffer (NEB, Cat#M0297S),

5 μL 1mg/mL Proteinase K,

3 μL RNase H (NEB, Cat#M0297S),

2.5 μL 10% TritonX 100.

Following dA tailing, the cDNA in the FFPE sections were transferred into a gene expression slide with the transfer mix at 37℃ for 30 min using our home-made instrument (Supplementary Fig. 1) or the standard 10X CytAssist. The details were following:

a. The homemade instrument was placed in an oven set at a temperature of 37℃;
b. The gene expression slide was securely fixed onto the lower slider holder using a slide anchor, while the FFPE section was immobilized on the upper slider holder with a section anchor;
c. The prepared transfer mix was carefully applied onto the gene expression slide, and then the upper slide holder was smoothly

guided through the lift guide mechanism to maintain its horizontal position;

d. Subsequently, the elevating motor was activated, causing the upper slider holder to descend gradually under gravity until it aligned perfectly with both the gene expression slide and FFPE section;
e. A photograph of this alignment was captured using a camera;
f. After incubating for 30 minutes at 37℃, the elevating motor was turned on again to swiftly separate and remove the gene expression slide from the FFPE section for subsequent experiments.

6. **DNA extension in situ**

100 μL transfer mix:

72 μL dd$H_2O$,

10 μL 10X buffer (M20 Genomics, Cat#R20114124),

10 μL 10mM dNTP,

8 μL DNA Polymerase (M20 Genomics, Cat#R20114124).

The gene expression slide were incubated with the extension mix at 60℃ for 60 min.

7. **Library preparation**

98℃ 3 min

10/rest cycles of

98℃ 15 s

63℃ 20 s

72℃ 30 s

End cycles

72℃ 1 min

4℃ forever

Following DNA extension, the standard 10X Visium CytAssist library preparation was followed to generate cDNA and final sequencing libraries: The single cDNA containing the spatial barcode was eluted using 50 µl 0.08 M KOH (Millipore Sigma Cat # P4494-50ML) and 3 µl 1 M Tris-HCl pH 8.0. Amplify the mix using 10X Genomics pre-amplification mix for 10 cycles and cleanup by SPRIselect (10X Genomics, Cat # PN-1000521). The rest cycle number of determinations was performed by qPCR and final sequencing libraries was amplified by dual index primers. The libraries were then pooled and sequenced using the NovaSeq 6000 (Illumina).