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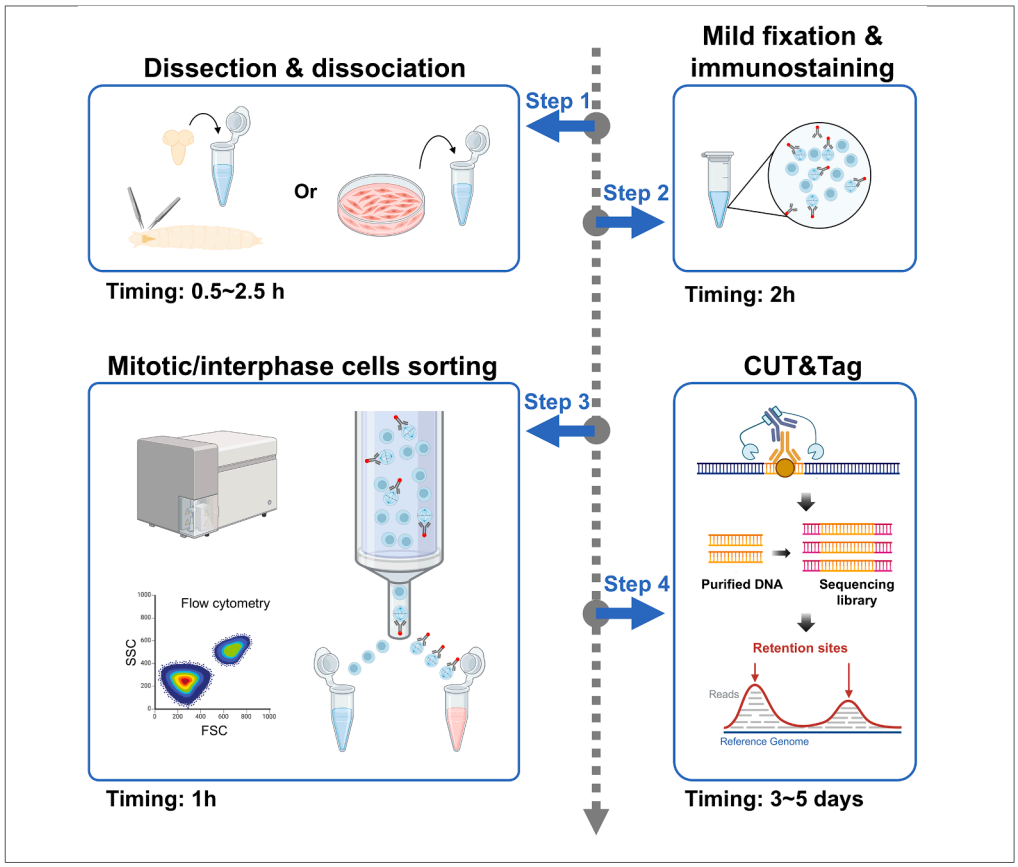
Protocol for identifying genomic binding sites of mitotic bookmarkers in *Drosophila* neural stem cells and cultured mammalian cells



Mitotic bookmarkers label key gene loci on highly condensed chromosomes to preserve cell fate memory across mitosis. Here, we present a protocol for identifying binding sites of mitotic bookmarkers (BISMIBs) in developing tissues and cultured cells. We describe a workflow for isolating mitotic and interphase cells from *Drosophila* larval brains and HEK293T cells independent of cell-cycle synchronization. We further detail procedures for low-input *in vivo* CUT&Tag (Cleavage Under Targets & Tagmentation) sequencing. This protocol is applicable for identifying binding sites for mitotically retained factors in developing tissues.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Yuying Shen, Jie Liu, Yan Song

yyshen@stu.pku.edu.cn (Y.S.)
yan.song@pku.edu.cn (Y.S.)

Highlights

Procedure for mild fixation and immunolabeling followed by flow cytometry and CUT&Tag

Isolation of mitotic cells from fly brains independent of cell-cycle synchronization

Identification of genomic binding sites of mitotic bookmarkers in developing tissues

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Protocol for identifying genomic binding sites of mitotic bookmarkers in *Drosophila* neural stem cells and cultured mammalian cells

Yuying Shen,[1,3,*] Jie Liu,[1] and Yan Song[1,2,4,*]

[1]State Key Laboratory of Membrane Biology, Ministry of Education Key Laboratory of Cell Proliferation and Differentiation, School of Life Sciences, Peking University, Beijing 100871, China

[2]Peking-Tsinghua Center for Life Sciences, Peking University, Beijing 100871, China

[3]Technical contact

[4]Lead contact

*Correspondence: yyshen@stu.pku.edu.cn (Y.S.), yan.song@pku.edu.cn (Y.S.)
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SUMMARY

Mitotic bookmarkers label key gene loci on highly condensed chromosomes to preserve cell fate memory across mitosis. Here, we present a protocol for identifying binding sites of mitotic bookmarkers (BISMIBs) in developing tissues and cultured cells. We describe a workflow for isolating mitotic and interphase cells from *Drosophila* larval brains and HEK293T cells independent of cell-cycle synchronization. We further detail procedures for low-input *in vivo* CUT&Tag (Cleavage Under Targets & Tagmentation) sequencing. This protocol is applicable for identifying binding sites for mitotically retained factors in developing tissues.

For complete details on the use and execution of this protocol, please refer to Shen et al.[1]

BEFORE YOU BEGIN

The following protocol outlines the precise steps required for the preparation of brain cell suspension from *Drosophila* larvae, dissociation of HEK293T cells, mild fixation and immunostaining, mitotic and interphase neural stem cell (NSC) and HEK293T cell sorting, and downstream CUT&Tag (Figure 1). The tissue used in this experiment is the third-instar larval brain of *Drosophila melanogaster*.

Preparation for dissection of *Drosophila* larval brain

Timing: 30 min

1. Collect about 150 third-instar *brat* (*brain tumor*), CD8-RFP; *PntP1*-Gal4 *Drosophila* larvae at 96 h after egg hatching. Larva gender is not distinguished.
2. Prepare the dissection station by arranging the following materials:
   a. Clean workbench.
   b. Stereoscope.
   c. Dissection tweezers.
   d. 1.5 mL low binding tubes.
   e. 1× PBS, complete Schneider's medium, and 75% ethanol.
3. Preheat dry bath at 30°C.
4. Precooling 4°C centrifuge.

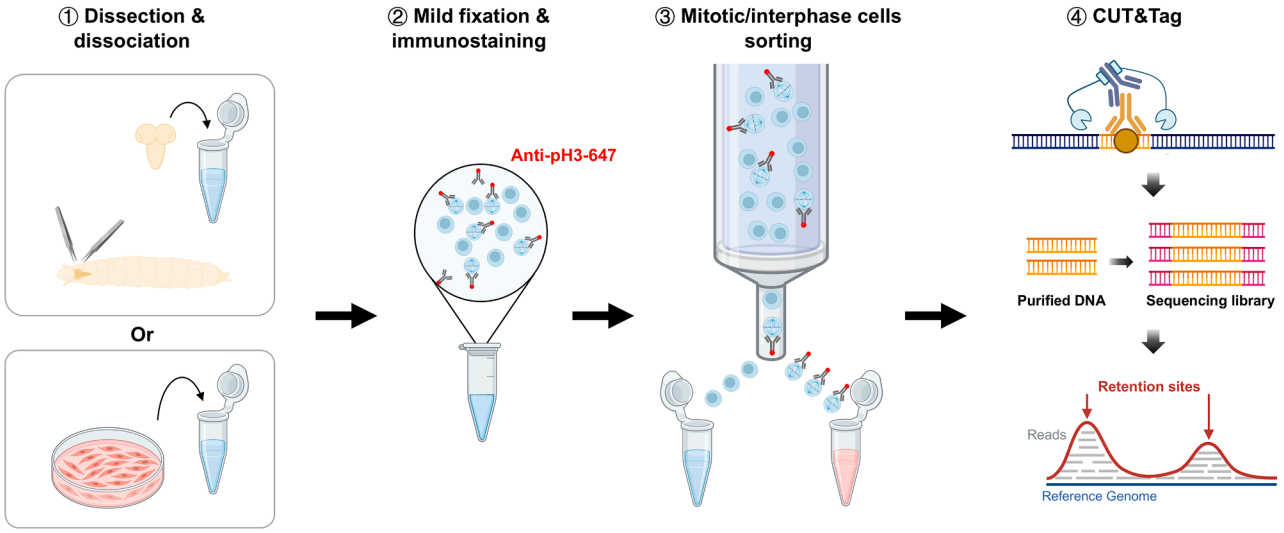

Figure 1. A workflow for identifying mitotic retention sites of bookmarkers independent of cell cycle synchronization

Preparation for dissociation of HEK293T cell

Timing: 2–3 days

5. Prepare 3 dishes (100 mm) of HEK293T cells with a growth confluency of 70–80%.
6. Preheat complete DMEM medium and 1× PBS at 37°C.
7. Preheat trypsin at 22°C-25°C.

Preparation for flow cytometer

Timing: 1.5 h

8. Prepare about 10 L 1× PBS as sheath solution.
9. Turn on the flow cytometer and set optimal parameters. Use a 100-μm nozzle for sorting NSCs and 80-μm nozzle for sorting HEK293T cells.
10. Turn on the sheath fluid cooling system.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse anti pH3-647	Abcam	Cat# ab196698
Chemicals, peptides, and recombinant proteins		
Collagenase, from *Clostridium histolyticum*	Sigma	Cat# C2674
Dulbecco's modified Eagle's medium (DMEM)	Gibco	Cat# C11995500BT
Fetal bovine serum (FBS)	Gibco	Cat# 10091-148; Lot# 2556131P
Fetal bovine serum (heat inactivated)	Sigma	Cat# F4135
Glycine	VWR Life Science	Cat# 0167
Insulin solution from bovine pancreas	Sigma	Cat# I0516
L-glutamine solution	Sigma	Cat# G7513
L-glutathione reduced	Sigma	Cat# G6013

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
MEM non-essential amino acids solution (100×)	Gibco	Cat# 11140-050
Papain, from papaya latex	Sigma	Cat# P4762
8% paraformaldehyde (PFA)	Electron Microscopy Sciences	Cat# 157-8
Penicillin-streptomycin	Gibco	Cat# 15140-122
Poly-D-lysine	Sigma	Cat# P6282
Schneider *Drosophila* culture medium	Gibco	Cat# 21720024
Triton X-100	Sigma	Cat# T8787
0.25% trypsin with EDTA	Gibco	Cat# 25200-072
VAHTS DNA clean beads	Vazyme	Cat# N411
Experimental models: Cell lines		
HEK293T	ATCC	CRL-1573
Experimental models: Organisms/strains		
Drosophila, *brat* [k06028]	Arama et al.[2]	N/A
Drosophila, UAS-CD8-RFP	This paper	N/A
Drosophila, *PntP1*-Gal4	Zhu et al.[3]	N/A
Critical commercial assays		
TruePrep index kit V2 for Illumina	Vazyme	Cat# TD202
Hyperactive universal CUT&Tag assay kit for Illumina	Vazyme	Cat# TD903
Software and algorithms		
bedtools (v.2.30.0)	Quinlan et al.[4]	https://github.com/arq5x/bedtools2
Bowtie2 (v.2.5.1)	N/A	https://bowtie-bio.sourceforge.net/bowtie2/index.shtml
ChIPseeker (v.1.34.1)	Yu et al.[5]	https://github.com/YuLab-SMU/ChIPseeker
clusterProfiler (v.4.8.2)	Yu et al.[6]	https://guangchuangyu.github.io/software/clusterProfiler/
deepTools (v.3.5.1)	Ramírez et al.[7]	https://github.com/deeptools/deepTools
FastQC (v.0.11.9)	N/A	https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
ggplot2 (v.3.4.2)	N/A	https://ggplot2.tidyverse.org/
GraphPad Prism 8	GraphPad	https://www.graphpad-prism.cn/
IGV browser	N/A	https://software.broadinstitute.org/software/igv/
IDR (v.2.0.4.2)	Li et al.[8]	https://github.com/nboley/idr
Macs2 (v.2.2.7.1)	Zhang et al.[9]	https://pypi.org/project/MACS2/
MultiQC (v.1.14)	Ewels et al.[10]	https://multiqc.info/
org.Dm.eg.db (v.3.16.0)	N/A	https://bioconductor.org/packages/org.Dm.eg.db/
Photoshop CC2015	Adobe	http://www.adobe.com
Picard (v.3.0.0)	N/A	https://broadinstitute.github.io/picard/
RStudio	RStudio	https://posit.co/products/open-source/rstudio/
R version 4.2.1	N/A	https://cran.r-project.org/
Samtools (v.1.6)	Li et al.[11]	https://www.htslib.org/
Trim Galore (v.0.6.10)	N/A	https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/
Other		
100 mm cell culture dishes	TrueLine	Cat# TR4002
LoBind tubes	Eppendorf	Cat# 0030108418
Magnetic rack	Vazyme	Cat# CM101
Tube 5 mL 12 × 75 mm RBtm w/strain	Falcon	Cat# 352235
Steriflip	Millipore	Cat# SCGP00525
Inverted confocal microscope (Leica TCS SP8)	Leica Microsystems	N/A
40×, 1.3 NA oil-immersion objective	Leica Microsystems	N/A
Aria SORP	BD	N/A

MATERIALS AND EQUIPMENT

Rinaldini's solution (R solution)		
Reagent	Final concentration	Amount
NaCl	140 mM	800 mg
KCl	2.7 mM	20 mg
NaH_2PO_4	0.42 mM	5 mg
$NaHCO_3$	11.9 mM	100 mg
Glucose	5.6 mM	100 mg
ddH_2O	N/A	100 mL
Total	N/A	100 mL

Rinaldini's solution can be prepared as a 10× solution and stored at 4°C for up to 3 months. Filter-sterilize the solution using the Steriflip.

Complete Schneider's medium		
Reagent	Final concentration	Amount
Fetal Bovine Serum (heat inactivated)	10% (vol/vol)	1 mL
Insulin solution from bovine pancreas	0.02 mg/mL	20 μL
Penicillin-Streptomycin	200 U/mL	0.2 mL
L-Glutamine solution	20 mM	1 mL
L-Glutathione	0.4 mg/mL	4 mg
Schneider *Drosophila* culture-medium	N/A	7.8 mL
Total	N/A	10 mL

Store at 4°C after filter-sterilization for no more than one week.

Complete DMEM medium		
Reagent	Final concentration	Amount
Fetal bovine serum	10% (vol/vol)	50 mL
Non-Essential Amino Acid	1% (vol/vol)	5 mL
DMEM medium	N/A	445 mL
Total	N/A	500 mL

The Complete DMEM medium is prepared in a cell culture hood. Store at 4°C for up to 3 months.

Dissociation solution		
Reagent	Final concentration	Amount
Papain	1 mg/mL	25 μL
Collagenase	1 mg/mL	25 μL
Complete Schneider's medium	N/A	450 μL
Total	N/A	500 μL

Prepare fresh for each experiment.

Glycine solution		
Reagent	Final concentration	Amount
Glycine	2.5 M	0.938 g
ddH_2O	N/A	5 mL
Total	N/A	5 mL

Store at 4°C after filter-sterilization for no more than 1 month.

PBST buffer		
Reagent	Final concentration	Amount
Triton X-100	0.1% (vol/vol)	0.1 mL
Sterilized 1× PBS	N/A	99.9 mL
Total	N/A	100 mL
Store at 25°C for up to 6 months.		

⚠ CRITICAL: PFA is highly toxic. Wear gloves and goggles and operate inside the fume hood. Triton X-100 is toxic. Please handle it with caution and ensure proper protective measures to avoid direct skin contact or inhalation.

STEP-BY-STEP METHOD DETAILS

Dissociation of *Drosophila* NSCs

Timing: 2.5 h

This step is adapted from Harzer et al.[12] to generate single cell suspension.

1. Gather third-instar larvae with the corresponding genotype and place them in a dish containing 5 mL of PBS.
2. Rinse the larvae once with PBS to remove any residual food particles.
3. Transfer the larvae into complete Schneider's medium and dissect the larval brains with forceps.
4. Transfer each brain into a 1.5 mL low-binding tube containing 500 μL R solution on ice.

⚠ CRITICAL: To maintain the tissue integrity of the dissected brains, ensure that the total dissection time does not exceed 60 min. Multiple people can perform dissection in parallel to ensure timely collection of adequate numbers of brain samples within 60 min.

5. After all the brains have settled at the bottom of the tubes, carefully remove the R solution and rinse the brains again with cold R solution.

⚠ CRITICAL: Be careful not to aspirate the brain tissue into the pipette tips, as it will stick to the plastic and cause sample loss.

⚠ CRITICAL: Visually check whether all the brains have sunken to the bottom. Do not let the brains dry out while removing the R solution—leave a small amount to cover them.

6. Remove the R solution and any floating materials (e.g., residual salivary glands).
7. Add 500 μL dissociation solution (for 50–200 brains) to the brains. Incubate the tubes for 30–50 min at 30°C in the thermomixer without shaking. Carefully mix the brain samples every 15 min by aspirating the solution and blowing with a pipette.

⚠ CRITICAL: Use a pipette tip to aspirate the upper part of the solution and gently blow once to avoid inhaling the brains. The brain samples will settle at the bottom of the tube afterward.

8. Remove the dissociation solution with a pipette and rinse twice with 200 μL cold PBS.
9. Dissociate the tissue by pipetting the brains in the cold PBS up and down for 40–60 times using a 200-μL pipette tip until the mixture becomes homogeneous.
10. Pass the cell suspension through a 35-μm mesh filter to remove debris.

Optional: At this step you can check cell viability, cell number, and cell density by Trypan blue staining and cell count.

Dissociation of HEK293T cells

Timing: 0.5 h

To identify the mitotic retention sites of bookmarkers in cultured cells, the following steps can be performed for cell dissociation. At 70–80% confluency, a high proportion of cells are in the mitotic phase.

11. Carefully aspirate the cell culture medium from the culture dish.
12. Add 5 mL 1× PBS to each dish to wash the cells. Slowly add the solution along the sidewall of the culture dish to prevent disruption of the cell layer, and carefully tilt the dish back and forth a few times.
13. Carefully discard the 1× PBS solution.
14. Add 1 mL preheated trypsin to each dish. Incubate at 37°C for 30–60 s. Gently swirl the container to ensure the trypsin fully covers the cell layer.

Note: The incubation time varies depending on the cell line used. If the cell dissociation rate is low, extend the incubation time. Lightly tapping the container can accelerate cell dissociation.

15. Add 5 mL preheated complete DMEM medium to each dish. Pipette repeatedly to create a uniform cell suspension.
16. Transfer the cell suspensions from three dishes into two 15 mL centrifuge tubes.
17. Centrifuge the tubes containing HEK293T cells at 300 × *g* for 8 min at 4°C.

Note: The centrifugation speed and time vary depending on cell type.

18. Pour off the supernatant, resuspend the pellet in 800 μL cold 1× PBS, and equally distribute the suspension into two 1.5 mL tubes.

Mild fixation and immunostaining of NSCs

Timing: 2 h

These steps label mitotic NSCs and prepare for cell sorting.

19. Add fresh 8% PFA to a final concentration 0.1% and flick the tubes gently with fingers until the solution appears homogeneous. Shake the tubes at a 20 rpm rotator for 7 min at 22°C–25°C.
20. Immediately quench fixation by introducing Glycine solution to a final concentration 0.125 M and leave at 22°C–25°C for 5 min.
21. Centrifuge the tubes at 500 × *g* for 7 min at 4°C.
22. After carefully discarding the supernatant, add 200 μL PBST to the pellet and shake at 20 rpm, 22°C–25°C for 7 min.
23. Centrifuge the tubes at 500 × *g* for 7 min at 4°C.
24. In the meantime, prepare 647-pH3 antibody solution: add 2 μL anti-pH3-647 mouse antibody (pH3 antibody conjugated with Alexa Fluor 647) in 200 μL cold 1× PBS. Keep the antibody solution on ice.
25. Withdraw the supernatant carefully, resuspend the pellet in 200 μL 647-pH3 antibody solution and shake at 20 rpm, 4°C for 60 min.
26. Centrifuge the tubes at 500 × *g* for 7 min at 4°C.

Optional: Carefully remove the supernatant. Resuspend the cell pellet with 500 μL cold PBS. Centrifuge the tubes at 500 × *g* for 7 min at 4°C. Discard supernatant after centrifugation.

27. Carefully withdraw the supernatant and resuspend the pellet with 200 μL cold 1× PBS.

Note: If more than 200 brains are dissected, moderately increase the amount of PBS.

Mild fixation and immunostaining of HEK293T cells

Timing: 2 h

These steps label mitotic HEK293T cells and prepare them for cell sorting.

28. Add fresh 8% PFA to a final concentration 0.1% and flick the tube gently with fingers until the solution appears homogeneous. Shake the tubes at a 20 rpm rotator for 7 min at 22°C–25°C.
29. Immediately quench fixation by adding Glycine solution to a final concentration 0.125 M and let stand at 22°C–25°C for 5 min.
30. Centrifuge the tubes at 300 × *g* for 7 min at 4°C.
31. Carefully remove the supernatant, add 800 μL PBST and shake at 20 rpm, 22°C–25°C for 7 min.
32. Centrifuge the tubes at 300 × *g* for 7 min at 4°C.
33. Prepare pH3 antibody solution: add 2 μL anti-pH3-647 mouse antibody (pH3 antibody conjugated with Alexa Fluor 647, Abcam) in 200 μL cold 1× PBS.
34. Remove the supernatant carefully, add 200 μL pH3-647 antibody solution.
35. Combine the cells from two tubes into one tube.
36. Shake at 20 rpm, 4°C for 60 min.
37. Centrifuge the tubes at 300 × *g* for 7 min at 4°C. Carefully withdraw the supernatant and wash the cell pellet again with 500 μL cold PBS.
38. Centrifuge the tubes again, carefully withdraw the supernatant before resuspending the pellet in 500 μL 1× PBS on ice.

Note: The volume of PBS can be adjusted according to the number of cells.

Note: Follow-up procedures are the same as for NSCs, unless otherwise stated. HEK293T cells were centrifuged with 300 × *g*.

Mitotic and interphase NSC sorting (same for HEK293T cells)

Timing: 1 h

These are the general steps for FACS (fluorescence-activated cell sorting).

39. Initiate the sorting process with flow cytometry sorter (e.g., Aria SORP).
40. Use 1.5 mL low-adhesion tubes as the collection tubes. Add 200 μL of complete Schneider's medium into the collection tube. (For HEK293T cells, complete DMEM medium is used as collection buffer). Wet the inner walls of the tube with the collection medium.

Alternatives: PBS can also be used instead of complete Schneider's medium or complete DMEM medium as collection buffer, but a 10% FBS buffer should be added. When isolating a limited quantity of cells, the FBS present in the collection liquid can prevent static electricity and facilitate the adsorption of cells into the collection buffer.

41. Prior to sorting, gently mix the cells by lightly tapping the tube a few times before placing the tube in the chilled sample holder.

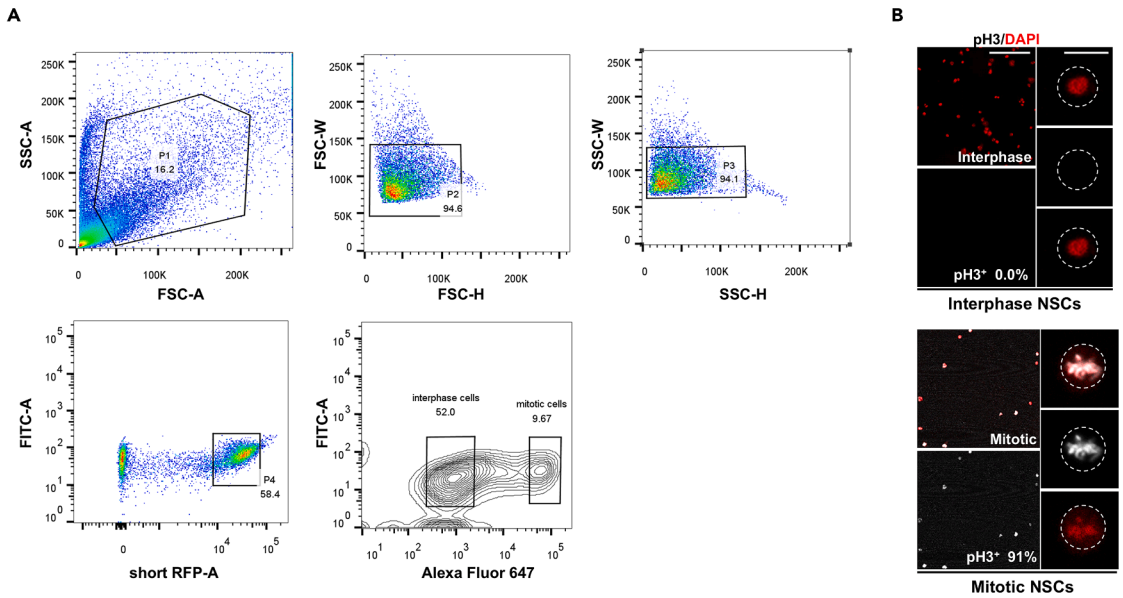

Figure 2. Mitotic and interphase NSCs sorting with flow cytometry

(A) The sorting of mitotic and interphase NSCs by flow cytometry is based on the fluorescence signal of RFP and pH3-647, which specifically labels mitotic NSCs.

(B) Purity of the interphase and mitotic NSCs after FACS purification. n=99 (interphase) and 102 (mitosis) respectively. Scale bar, 40 μm (left); 10 μm (right).

42. Analyze the cell sample, and collect data on at least 100,000 events to obtain profile about all cell populations present. Troubleshooting 1.
43. Plot side scatter (SSC) and forward scatter (FSC).
44. Sort cells based on CD8-RFP^{+} and large size (> 8 μm in diameter) for 647^{+} mitotic NSCs, and 647^{-} for interphase NSCs (Figure 2). Troubleshooting 2.
45. For purity examination, place unsorted, mitotic or interphase NSCs onto a poly-D-lysine coated cover glass, add 0.1% (vol/vol) DAPI, keep on ice for 30 min. Avoid exposure to light and disturbing the cell sedimentation. Confirm the population of sorted NSCs is pure under confocal microscopy (Figure 2B). Troubleshooting 3.

Note: The volume of PBS can be adjusted according to the number of cells.

CUT&Tag-seq

Timing: 2 days

The CUT&Tag experiments are conducted using the Hyperactive Universal CUT&Tag Assay Kit for Illumina: https://bio.vazyme.com/product/450.html. Library amplification and purification are carried out following the standard protocol with the TruePrep Index Kit V2 for Illumina with the standard protocol: https://bio.vazyme.com/product/187.html.

Note: To ensure reliable data on protein binding during mitosis, it is recommended that the number of mitotic NSCs used in the CUT&Tag assay exceed 50,000. If the abundance of the detected protein is extremely low, more cells are needed.

46. Centrifuge the mitotic NSC and interphase NSCs at 500 × *g* for 7 min at 4°C. Carefully discard the supernatant and resuspend the cells in 500 μL of Wash buffer.

Table 1. Reagents of a single 50 μL PCR reaction

Reagent	Amount
DNA fragments	15 μL
2× CAM	25 μL
N5 primer	5 μL
N7 primer	5 μL
ddH_2O	50 μL

Note: The subsequent steps are carried out at 22°C-25°C.

47. Incubate cells with ConA-coated magnetic beads on a 20 rpm shaker at 22°C–25°C for 10 min.

Note: After adding ConA-coated magnetic beads, avoid vigorous shaking or pipetting to prevent cell detachment. It is normal for some magnetic beads to gather during incubation.

48. Remove the supernatant carefully and incubate the sample with primary antibody in 50 μL antibody buffer (1:100) at 4°C for 8–72 h.

Pause point: 8–72 h for incubation at 4°C.

Note: The concentration of antibodies (e.g. IgG) in the control group should be the same as that in the experimental group. A blank group without the addition of primary antibody can also be used as a negative control.

49. Discard the supernatant carefully and incubate the sample with secondary antibody in 200 μL Dig-wash buffer (1:100) on a 20 rpm shaker for 60 min.
50. Rinse the samples three times with 200 μL Dig-wash buffer: Instantaneously centrifugate the sample. Place the sample on the magnetic rack. After the solution is clarified (30 s–2 min), discard the supernatant.
51. Prepare the hyperactive pA/G-transposon containing buffer by mixing 2 μL pA/G-Tnp with 98 μL Dig-300 buffer.
52. Add 100 μL hyperactive pA/G-transposon containing buffer and incubate at 22°C–25°C for 60 min.
53. Rinse the samples three times with 200 μL Dig-300 buffer.
54. In order to activate transposase, add 50 μL TTBL buffer and incubate the sample at 37°C in the Thermomixer for 60 min.
55. Add 100 μL buffer L/B, 5 μL Proteinase K and 20 μL DNA Extract beads to the sample. Vortex and incubate at 55°C in dry bath for 10 min. Carefully remove the supernatant.
56. Rinse the beads once with 200 μL of buffer WA and twice with 200 μL of buffer WB. Discard the supernatant.
57. Dry the magnetic beads. Elute the DNA fragments with 15–20 μL instead of the recommended 22 μL distilled H_2O.

Table 2. PCR cycling conditions

Temperature	Time	Cycles
72°C	3 min	1
95°C	3 min	1
98°C	10 s	16–21
60°C	5 s	
72°C	1 min	1
4°C	forever	

Pause point: DNA fragment can be preserved at −20°C for extended periods. Avoid repeated freeze-thaw cycles.

58. Library amplification: An overview of the reagents needed for PCR reaction are listed in Table 1. The PCR procedure Settings are listed in Table 2, referring to the standard protocol, e.g. TD202, Vazyme.

If the amount of the product is too little for subsequent sequencing, see troubleshooting 4 to modify the cell input and the number of amplification cycles.

59. Bring VAHTS DNA Clean Beads to 22°C–25°C. Perform size selection to obtain appropriate fragments: mix 100 μL DNA Clean Beads with 50 μL of PCR product and incubate at 22°C–25°C for 10 min.

Note: The VAHTS DNA Clean Beads need to be balanced at 22°C–25°C for 30min in advance. DNA Clean Beads are sticky, use a pipette to make sure you get enough volume and add slowly.

60. Discard the supernatant and rinse the beads twice with fresh 80% (vol/vol) ethanol on magnetic rack.
61. Dry the magnetic beads. Elute the DNA fragments with 15–20 μL distilled H_2O.
62. The final libraries are sequenced as 150-bp paired-end reads using a Novaseq platform, achieving raw sequencing depths ranging from 10 to 20 million read pairs.

Data analyses

Timing: 1–3 days

This pipeline allows for: 1) Quality Control & Adapter trimming; 2) Alignment; 3) Post-Alignment filtering; 4) Visualization; 5) Peak calling and 6) Annotation of peaks and other downstream analysis. Main scripts used for bioinformatic data analyses can be downloaded at:

https://github.com/JieLiu2023/mitoticCUT-Tag_2024.

63. Quality control & Adapter trimming.
    a. Preliminary quality control: Monitor the quality of raw FASTQ reads with FastQC and MultiQC.
    b. Adapter trimming: Raw FASTQ reads are trimmed with Trim Galore (with parameters –paired –quality 20 –stringency 3 -j 4) to eliminate low-quality bases and adapters.
64. Alignment. After preliminary quality control and adapter trimming, paired-end reads are aligned to *Drosophila melanogaster* (or *Homo sapiens*) reference genome using Bowtie2.
    a. Dm6: Pre-built Bowtie2 index for dm6 genome.
    b. GRCh38: Pre-built Bowtie2 index for GRCh38 genome. Alignment parameters are listed below.

```
bowtie2 --local --very-sensitive-local --no-mixed --no-discordant --phred33 -I 10 -X 700 -p 8 -x
${dm6_or_GRCh38_bowtie2_index}
```

65. Post-alignment filtering.
    a. Label and remove duplicate reads with Picard SortSam and Picard MarkDuplicates.
    b. Further filtering. Reads unmapped or with a low mapping quality score < 10 are removed with samtools view (samtools view -q 10 -F 0x04).

66. Visualization.
    a. Generate bigWig files. Generate bigWig files using bamCoverage –normalizeUsing RPKM –extendReads.
    b. Generate heatmaps. Use deeptools to generate enrichment profile of peaks around transcription start sites (TSSs) or peak summits (described below).
67. Peak Calling. This pipeline is inspired by ICEBERG algorithm.[13]
    a. Using samtools merge to pool Bam files of biological replicates, and then randomly downsample to generate 3 pseudo-replicates with same read depth using picard DownsampleSam. Parameters are listed below.

```
picard DownsampleSam --ACCURACY 1.0E-5 --RANDOM_SEED ${random_seed} --STRATEGY ConstantMemory
```

    b. Performing peak calling on each pseudo-replicate separately with Macs2 callpeak (with parameters -f BAMPE -q = 0.05).
    c. Perform an Irreproducible Discovery Rate test on every pair of narrowPeak files from pseudo-replicates to detect signals with high consistency and reproducibility. The signals that pass the IDR test (with an IDR threshold defined as 0.5, which means scaled IDR value in 5th field > 125) are kept and merged using bedtools to generate "mitosis/interphase peak sets".
    d. Identify "interphase-only", "mitosis-only" and "overlap (mitotic retention)" peaks with bedtools intersect.
68. Annotation of peaks and other downstream analysis. Final peak signals are loaded into R. ChIPseeker and clusterProfiler are the major R packages used in further analysis such as peak annotation and GO analysis. Troubleshooting 5.

EXPECTED OUTCOMES

The main goal of this protocol is to isolate and purify mitotic and interphase cells from developing tissues or cultured mammalian cells independent of cell cycle synchronization, enabling the identification of genomic binding sites of proteins or modifications during both mitotic and interphase stages. With mild fixation and immunolabeling with pH3 antibody conjugated to fluorophores, flow cytometry can segregate the mitotic and interphase cells with purity of more than 90% (Figure 2B).[1]

Therefore, when combined with low-input CUT&Tag-seq, this pipeline identifies the mitotic retention sites of bookmarkers in developing tissues, facilitating the exploration of their biological functions and molecular mechanisms.

The mild fixation-pH3 staining protocol for isolating mitotic cells is widely applicable beyond *Drosophila* type II NSCs and HEK293T cells. For instance, the combination of this method with *ase*-Gal4 > CD8-RFP (CD8-RFP driven by type I NSC-specific Gal4) can be used to isolate mitotic versus interphase *Drosophila* type I NSCs as well. With minor adjustments (e.g., tissue dissociation procedures), this method can also be adapted for sorting interphase and mitotic cells across diverse tissue types and cell lines.

LIMITATIONS

Since wild type *Drosophila* larval brains contain only 8 type II NSCs per brain lobe, we perform *in vivo* CUT&Tag using type II NSCs derived from *brat* (*brain tumor*) mutant larvae. The *brat* mutant larval brains contain up to 1,000 type II NSCs per brain lobe, providing a large and pure pool of NSCs ideal for omics analysis.[14,15]

TROUBLESHOOTING

Problem 1 (step 42)

Cell adhesion or shrinkage in cell sorting.

Table 3. Regarding cell input and number of amplification cycles

Cell input	Amplification cycle
50,000	20 cycles
100,000	18 cycles
200,000	16 cycles

Potential solution

- The cell adhesion is owing to insufficient enzymatic digestion. Prepare enzyme stocks fresh and avoid multiple freeze-thaw cycles. If necessary, increase either the enzyme concentration or the incubation duration. Ensure that the final enzyme concentration does not exceed 2 mg/mL and the incubation time remains within 1 h.
- When there are enough brains for digestion, gently pipette and triturate the sample during enzymatic digestion.
- The tubes are flicked after centrifugation, rather than being blown straight with a low binding pipette tip.
- Ensure complete resuspension after each centrifugation step.
- If sufficient brain tissue is available, you can filter the cells again with a 35-μm cell strainer before the flow sorter.
- The brain dissection process should be strictly controlled within 60 min, otherwise the cells may not be in a good state and begin to die.
- Perform mechanical disruption as gently as possible.

Problem 2 (step 44)

The proportion of NSCs is low.

Potential solution

- Avoid cell death during digestion, mild fixation and immunostaining. Refer to troubleshooting problem 1.
- Reduce the centrifugation speed and use a larger nozzle.

Problem 3 (step 45)

The sorted mitotic/interphase NSCs are mixed with progenitors or neurons.

Potential solution

If impurities are detected in the isolated mitotic/interphase NSCs, the gate plotting cell size can be defined more stringently during the sorting process. NSCs are larger than 8 μm in diameter, while progenitors and neurons are much smaller.

Problem 4 (step 58)

Cell input and amplification cycles.

Potential solution

Cell input requirements vary depending on cell type, antibody specificity, and abundance of target protein abundance. The PCR cycle numbers corresponding to the amount of cell input are shown in Table 3.

During PCR amplification, avoid excessive amplification cycles while maintaining library yield. Excessive cycles can lead to over-amplification, increased bias, increased duplication rates, and other adverse effects.

Problem 5 (step 68)

The binding peaks of the target protein exhibit a low signal-to-noise ratio.

Potential solution

- Ensure the primary antibody used in CUT&Tag has high specificity.
- It is recommended to use ChIP-grade primary antibodies. If ChIP-grade antibodies are unavailable, consider testing antibodies validated for immunofluorescence (IF).
- This may result from low protein abundance or few binding sites. Increase the number of cells used in CUT&Tag to obtain sufficient reads.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yan Song (yan.song@pku.edu.cn).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Yuying Shen (yyshen@stu.pku.edu.cn).

Materials availability

This protocol does not include unique materials.

Data and code availability

This protocol does not include data sets.

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AUTHOR CONTRIBUTIONS

Conceptualization, Y. Song and Y. Shen; investigation, Y. Shen, J.L., and Y. Song; writing – original draft, Y. Shen; writing – review and editing, Y. Song; funding acquisition, Y. Song; supervision, Y. Song.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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