Photo-isolation chemistry (PIC) enables isolation of transcriptome information from locally defined areas by photo-irradiation. Here, we present an optimized PIC protocol for formalin-fixed frozen and paraffin mouse sections and fresh-frozen mouse sections. We describe tissue section preparation and permeabilization, followed by in situ reverse transcription using photo-caged primers. We then detail immunostaining and UV-mediated uncaging to the target areas, followed by linear amplification of uncaged cDNAs, library preparation, and quantification. This protocol can be applied to various animal tissue types.
Photo-isolation chemistry for high-resolution and deep spatial transcriptome with mouse tissue sections

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SUMMARY
Photo-isolation chemistry (PIC) enables isolation of transcriptome information from locally defined areas by photo-irradiation. Here, we present an optimized PIC protocol for formalin-fixed frozen and paraffin mouse sections and fresh-frozen mouse sections. We describe tissue section preparation and permeabilization, followed by in situ reverse transcription using photo-caged primers. We then detail immunostaining and UV-mediated uncaging to the target areas, followed by linear amplification of uncaged cDNAs, library preparation, and quantification. This protocol can be applied to various animal tissue types. For complete details on the use and execution of this protocol, please refer to Honda et al. (2021).

BEFORE YOU BEGIN
Photo-isolation chemistry (PIC) is a spatial transcriptome method for locally defined areas with the advantages of high spatial resolution and detection depth, ranging from large to small areas such as brain fields, embryonic tissues, single cells, and intracellular structures. PIC comprises the following five processes: (1) Sections are prepared from fresh or fixed tissues and permeabilized with HCl or Tris-EDTA (TE); (2) mRNAs are reverse-transcribed in situ with photo-caged primers; (3) the regions of interest (ROIs) are labeled by immunostaining and/or nuclear staining; (4) cDNA-conjugated primers are uncaged by photo-irradiation; (5) uncaged cDNAs are selectively amplified by in vitro transcription (IVT) before sequencing. Once tissue sections are prepared, two days are required to prepare sequencing libraries with standard molecular biology equipment and expertise.

Tissue preparation
The efficiency of in situ reverse transcription (RT) is best with fresh-frozen sections, followed by formalin-fixed frozen and paraffin sections (Figure 1A). However, formalin-fixed tissues have greater advantages in preserving tissue morphologies and preventing the dissipative loss of marker molecules for the visualization of ROIs, such as immunostaining and reporter protein antigens. In our experience, proteins that are less interactive with other molecules, such as GFP, are lost easily and need to be fixed (Figure 1B), whereas transcription factors and the proteins composing liquid-liquid phase separation are often detected without fixation. Furthermore, because RNAs are bound by various proteins and covalently cross-linked by formalin fixation, a permeabilization step is necessary to eliminate these interferences before in situ RT. In our experience, briefly soaking
the sections in HCl solution or heating them in TE solution is effective for fresh-frozen sections, whereas only the latter is effective for formalin-fixed tissues. Therefore, with a tissue for the first trial, both unfixed and fixed frozen sections were prepared and permeabilized with HCl or TE before immunostaining for ROI labeling (Figure 1A). If ROIs can be identified simply by nuclear staining, the fresh-frozen section is the best choice. If only paraffin sections were available, permeabilization with TE alone was tested. Therefore, the optimal conditions should be selected by evaluating the balance of tissue morphology, preservation of marker antigens, and efficiency of in situ RT.

**Synthesis of caged RT primer**

Purchase 50 μmol of 6-nitropiperonyloxymethyl NPOM-caged dT-CE phosphoramidite (Glen Research; #10-1534-95; Young et al., 2009) per construct and send it to the oligo house to synthesize caged RT primers, with the sequence shown in Figure 2A, by which at least 2 O.D. (= 66 μg) of the caged RT primers will be obtained. When caged RT primers with various barcode sequences, multiple samples can be pooled after photo-irradiation and cell lysis, thus reducing the cost of library synthesis for multiple samples.

△ CRITICAL: HPLC purification must not be ordered because UV is used for size evaluation and uncages NPOM moieties; rather, an oligonucleotide purification cartridge (OPC) grade should be ordered for purification. It is preferable to order it as a dried product.
in a light-shielded tube. After receiving, the product can be stored at $-20^\circ$C in light-shielded storage even when dissolved in 500 ng/μL with nuclease-free water. In later experiments, samples should be shielded from light during extended incubation times (Figure 3A), such as in situ RT and antibody reactions, but other than that, it is acceptable to perform the experiment under normal fluorescent light.

**Examination of irradiation time and cell number**

Because NPOM moieties are dissociated at 365 nm as their maximum absorption wavelength, they can be uncaged by the light path and filter cubes often used to excite DAPI and Hoechst under a
A. Incubation in the light-shielded humidified chamber

B. Washing section

C. Sealing with coverslip

D. Incubation under the controlled temperature

E. Removing coverslip

A paper towel soaked with PBS
standard fluorescence microscope, but the light emitted from the objective lens is limited to a circular shape (Figure 2B). To narrow down the irradiation area, it is better to use a higher magnification objective lens. For example, the Leica DM6B can emit a 250 μm excitation light with a 100 x objective lens and can be narrowed down to 16 μm using fluorescent field diaphragms, which are usually equipped with a standard fluorescence microscope. In contrast, a Digital mirror device (DMD) is appropriate for emitting light of an arbitrary shape according to the drawings on a computer (Figure 2B). We used Mightex Polygon1000-G, which has an excellent resolution by integrating a million micro-mirrors. Polygon1000-G can be extrapolated to upright or inverted microscopes from Leica, Zeiss, Olympus, and Nikon. We used a Leica DM6B upright microscope by mounting the Polygon1000-G between the lens barrel and the epi-illuminator module (Figure 2C). The light source of the DMD was separated from that originally supplied in the microscope to excite the probes for immunofluorescence (Figure 2D). The light source appropriate for the DMD-assisted uncaging is a high-power LED emitting around 365 nm, for which we used a product from Prizmatix (UHP-F-365 LED; 3 W). The above combination of microscope, DMD, and light source we have adopted is sufficient for uncaging with up to 3 min of irradiation. With other combinations, optimization of uncaging conditions is required, which can be easily tested using qPCR without sequencing. For instance, in our first setup, brain sections from adult mice were subjected to in situ RT with caged RT primers, and DMD-assisted UV irradiation was performed for 0, 10, 30, 60, 180, or 300 s with a 10 x objective at maximum light power. Subsequently, tissue lysates were analyzed using TaqMan qPCR with primers against the T7 promoter and Gapdh 3' UTR (Figure 2E), and sufficiently detected with photo-irradiation for longer than 3 min (Figure 2F).

Library synthesis, sequencing, and data analysis
After photo-irradiation, library synthesis in PIC method is largely based on the workflow CEL-seq2 (Hashimshony et al., 2016), one of the most sensitive single-cell RNA-seq technologies. The total tissue lysate was collected and purified. Second strand DNA was synthesized using the nick translation method using RNase H, DNA polymerase I, and a DNA ligase mix, by which only the cDNAs derived from the photo-irradiated region are filled-in to the 5' end of the T7 promoter, thereby allowing the cDNAs to be linearly amplified as RNAs (aRNAs) via IVT reaction. The libraries were further reverse-transcribed, followed by paired-end sequencing on the Illumina platform (Read 1: UMI and barcode, Read 2: cDNA). The given sequences were separated by the sample barcodes with UMI-tools and mapped to the reference genome using HISAT2. featureCounts and UMI-tools were used to generate UMI count data assigned to genes, and DESeq2 was used to extract DEGs.

Institutional permissions
All research and animal care procedures were approved by Kyoto University Graduate School of Medicine Institutional Animal Care.
### REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Tris-EDTA buffer solution (TE buffer; pH 8.0) | Nacalai Tesque | Cat#32739-31 |
| Triton X-100 | Nacalai Tesque | Cat#35501-02 |
| 5 M HCl | Nacalai Tesque | Cat#7647-01-0 |
| 1 M Tris-HCl (pH 8.0) | Nacalai Tesque | Cat#06938-15 |
| 0.1% tTBS (10× TBST; pH 7.4) | Nacalai Tesque | Cat#12750-81 |
| 0.1 M DTT | Thermo Fisher Scientific | Cat#YO0147 |
| Tween-20 | Nacalai Tesque | Cat#28353-85 |
| Proteinase K solution (20 mg/mL) | Kanto Chemical Co., Inc. | Cat#34060-96 |
| 5 M NaCl | Nacalai Tesque | Cat#06900-14 |
| Polyethylene glycol 8,000 (50% w/v) | Nacalai Tesque | Cat#26065-54 |
| Potassium Acetate | Nacalai Tesque | Cat#28404-15 |
| 0.5 M EDTA (pH 8.0) | Nacalai Tesque | Cat#06894-85 |
| Ethanol | Nacalai Tesque | Cat#14713-95 |
| NPOM-caged-dT-CE Phosphoramidite | Glen Research | Cat#10-1534-95 |

### Critical commercial assay

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| SuperScript II reverse transcriptase (200 U/µL) | Thermo Fisher Scientific | Cat#18064071 |
| 5x First Strand Buffer | Thermo Fisher Scientific | Cat#Y00146 |
| RNaseOUT (40 U/µL) | Thermo Fisher Scientific | Cat#10777019 |
| dNTP (10 mM each) | New England Biolabs | Cat#N0447L |
| Qiagen MiniElute PCR Purification kit | QIAGEN | Cat#28006 |
| 5x Second-Strand Buffer | Thermo Fisher Scientific | Cat#10812014 |
| E. coli DNA polymerase I (10 U/µL) | Thermo Fisher Scientific | Cat#18010025 |
| E. coli DNA ligase (10 U/µL) | Thermo Fisher Scientific | Cat#18052019 |
| RNase H (2 U/µL) | Thermo Fisher Scientific | Cat#18021071 |
| MEGAscript T7 Transcription Kit | Thermo Fisher Scientific | Cat#AM13345 |
| ATP (from the MEGAscript T7 Transcription Kit) | Thermo Fisher Scientific | Cat#B8114GS |
| GTP (from the MEGAscript T7 Transcription Kit) | Thermo Fisher Scientific | Cat#B8134GS |
| CTP (from the MEGAscript T7 Transcription Kit) | Thermo Fisher Scientific | Cat#B8124GS |
| UTP (from the MEGAscript T7 Transcription Kit) | Thermo Fisher Scientific | Cat#B8144GS |
| 10× T7 reaction buffer (from the MEGAscript T7 Transcription Kit) | Thermo Fisher Scientific | Cat#B8156GS |
| 10× Enzyme Mix (from the MEGAscript T7 Transcription Kit) | Thermo Fisher Scientific | Cat#B2720GS |
| TURBO DNase (from the MEGAscript T7 Transcription Kit) | Thermo Fisher Scientific | Cat#B2238GS |
| ExoSAP-IT Express PCR Product Cleanup | Thermo Fisher Scientific | Cat#75001.1.ML |
| AMPure XP beads | Beckman Coulter | Cat#A63881 |
| RNAClean XP beads | Beckman Coulter | Cat#A63987 |
| Phusion High-Fidelity PCR Master Mix | New England Biolabs | Cat#M0531L |
| High Sensitivity DNA kit | Agilent Technologies | Cat#506704626 |
| Ribonuclease A solution (10 mg/mL) | Nacalai Tesque | Cat#30100-31 |
|Blocking One-P | Nacalai Tesque | Cat#05999-84 |
| Nuclear Violet LCS1 | AAT Bioquest | Cat#17543 |
| PhiX Control v3 | illumina | Cat#FC-110-3001 |
| SlowFade Diamond | Thermo Fisher Scientific | Cat#S36963 |

### Experimental models: Organisms/strains

| Mouse: Slc:ICR, male, 8 weeks old | Charles River Laboratories | N/A |

### Biological samples

| Adult mouse brains (Slc:ICR, male, 8 weeks old) | Charles River Laboratories | N/A |

### Oligonucleotides

| NPOM-caged RT primer: GCCCGTAA TACGACTCATAAGGitttGAGtGt ACAGTCGAGACTCANNNNNNCTGT ACtttttttttttttttttttttttttttt | Honda et al. (2021) |
|-----------------------------------------------|------------------------|

| Read2+N6 primer: GCCCTTGGACCCG AGAATTCANNNNNNN | Honda et al. (2021) |
|-----------------------------------------------|------------------------|

| Read1 Index primer: AATGATACGCGCA CCACCCAGATCTACAC[AATGAGTT] GTTCAGAGTTCTACAGTCCGA | Honda et al. (2021) |

Replace [AATGAGTT] with an appropriate index.
### MATERIALS AND EQUIPMENT

#### Blocking solution

| Reagent       | Final concentration | Amount   |
|---------------|---------------------|----------|
| Blocking One-P | 0.5x                | 0.5 mL   |
| 1x TBST       | 0.5x                | 0.5 mL   |
| Total         | n/a                 | 1 mL     |

Freshly prepare before use.

#### Beads binding buffer

| Reagent                | Final concentration | Amount |
|------------------------|---------------------|--------|
| 50% w/v polyethylene glycol 8,000 | 20%                | 4 mL   |
| 5 M NaCl               | 2.5 M               | 5 mL   |
| Nuclease-free water    | n/a                 | 1 mL   |
| Total                  | n/a                 | 10 mL  |

This can be stored at room temperature (23°C–25°C) for at least 1 year.
### Fragmentation Buffer

| Reagent                          | Final concentration | Amount   |
|---------------------------------|---------------------|----------|
| 1 M KOAc                        | 0.5 M               | 5 mL     |
| 1.5 M MgOAc                     | 0.15 M              | 1 mL     |
| 1 M Tris-HCl (pH 8.0)           | 0.2 M               | 2 mL     |
| Nuclease-free water             | n/a                 | 2 mL     |
| **Total**                       | n/a                 | **10 mL**|

This can be stored at room temperature (23°C–25°C) for at least 1 year.

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### Read1 and 2 Index primer (10 μM each)

| Reagent                          | Final concentration | Amount   |
|---------------------------------|---------------------|----------|
| 100 μM Read1 Index primer       | 10 μM               | 10 μL    |
| 100 μM Read2 Index primer       | 10 μM               | 10 μL    |
| Nuclease-free water             | n/a                 | 80 μL    |
| **Total**                       | n/a                 | **100 μL**|

This can be stored at −20°C for at least 1 year.

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### NPOM-caged RT primer mix

| Reagent                          | Final concentration | Amount per section |
|---------------------------------|---------------------|--------------------|
| Nuclease-free water             | n/a                 | 5 μL               |
| NPOM-caged RT primer (500 ng/μL)| 25 ng/μL            | 0.5 μL             |
| dNTP mix (10 mM each)           | 0.5 mM              | 0.5 μL             |
| **Total**                       | n/a                 | **6 μL**           |

Freshly prepare before use.

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### First strand mix

| Reagent                          | Final concentration | Amount per section |
|---------------------------------|---------------------|--------------------|
| 5X First Strand Buffer          | 1x                  | 2 μL               |
| 0.1 M DTT                       | 0.01 M              | 1 μL               |
| RNaseOUT (40 U/μL)              | 2 U/μL              | 0.5 μL             |
| SuperScript II Reverse Transcriptase (200 U/μL) | 10 U/μL | 0.5 μL |
| **Total**                       | n/a                 | **4 μL**           |

Freshly prepare before use.

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### Second-strand mix

| Reagent                          | Final concentration | Amount per tube |
|---------------------------------|---------------------|-----------------|
| 5X First Strand Buffer          | 0.5x                | 2 μL            |
| 5X Second-Strand Buffer         | 0.5775x             | 2.31 μL         |
| dNTP mix (10 mM each)           | 0.115 mM            | 0.23 μL         |
| *E. coli* DNA Polymerase I (10 U/μL) | 0.15 U/μL  | 0.3 μL         |
| *E. coli* DNA Ligase (10 U/μL)  | 0.04 U/μL           | 0.08 μL         |
| RNase H (2 U/μL)                | 0.008 U/μL          | 0.08 μL         |
| **Total**                       | n/a                 | **5 μL**        |

Freshly prepare before use.
IVT mix

| Reagent                        | Final concentration | Amount per tube |
|--------------------------------|---------------------|-----------------|
| ATP                            | 7.5 mM              | 1.6 µL          |
| GTP                            | 7.5 mM              | 1.6 µL          |
| CTP                            | 7.5 mM              | 1.6 µL          |
| UTP                            | 7.5 mM              | 1.6 µL          |
| 10× T7 reaction buffer         | 1 x                 | 1.6 µL          |
| 10× Enzyme Mix                 | 1 x                 | 1.6 µL          |
| Total                          | n/a                 | 9.6 µL          |

Freshly prepare before use.

aRNA–N6 primer mix

| Reagent                        | Final concentration | Amount per tube |
|--------------------------------|---------------------|-----------------|
| aRNA (step 83)                 | n/a                 | 4.5 µL          |
| Read2+N6 primer (250 ng/µL)    | 25 ng/µL            | 1 µL            |
| dNTP mix (10 mM each)          | 0.5 mM each         | 0.5 µL          |
| Total                          | n/a                 | 6 µL            |

Freshly prepare before use.

Reverse transcription mix

| Reagent                        | Final concentration | Amount per tube |
|--------------------------------|---------------------|-----------------|
| 5× First Strand Buffer         | 1 x                 | 2 µL            |
| 0.1 M DTT                      | 0.01 M              | 1 µL            |
| RNaseOUT (40 U/µL)             | 2 U/µL              | 0.5 µL          |
| SuperScript II Reverse Transcriptase (200 U/µL) | 10 U/µL | 0.5 µL |
| Total                          | n/a                 | 4 µL            |

Freshly prepare before use.

Library PCR mix

| Reagent                        | Final concentration | Amount per tube |
|--------------------------------|---------------------|-----------------|
| Nuclease-free water            | n/a                 | 10.4 µL         |
| Read1 and 2 - Index primer (10 µM each) | 0.8 µM each | 3.6 µL         |
| Phusion High-Fidelity PCR Master Mix (2×) | 1 x | 22.5 µL |
| Secondary RT products (step 89) | n/a                 | 8.5 µL          |
| Total                          | n/a                 | 45 µL           |

Freshly prepare before use.

STEP-BY-STEP METHOD DETAILS

Post-fixation of tissue sections

© Timing: 15–80 min

Before performing PIC experiments, prepare fresh-frozen sections or formalin-fixed frozen or paraffin sections at a thickness of 10 µm on MAS-coated glass slides. When using PFA and D-limonene, work in a fume hood.

1. The tissues are hydrated, washed, and fixed using PFA solution according to the following protocols for frozen (a) or paraffin (b) sections:
CRITICAL: The liquid volume of PBS or PFA dropped on the sections depends on the size of the hydrophobic barrier surrounded by PAP PEN. For a 1 × 1 cm hydrophobic barrier, a volume of 50 μL of fluid is optimal to fully cover the sections and prevent leakage from the barrier. The following protocols describe a 1 × 1 cm hydrophobic barrier. Use an aspirator with a fine needle to remove the liquid.

a. Frozen sections (= 15 min).
   i. Drop and aspirate 50 μL of PBS onto the section twice to remove the OCT compound (Figure 3B).
   ii. Drop 50 μL of 4% PFA in PBS onto the section and incubate at RT for 10 min.
   iii. Aspirate the PFA solution and wash tissue sections twice with 50 μL of PBS.

b. Paraffin sections (= 80 min).
   i. Immerse the sections in a glass staining jar containing D-Limonene and incubate at RT for 5 min.
   ii. Repeat step (i) two more times with a separate glass staining jar.
   iii. Immerse sections in a glass staining jar containing ethanol (96%–100% [v/v]) and incubate at RT for 5 min.
   iv. Repeat step (iii) two more times with a separate glass staining jar.
   v. Immerse the sections in a glass staining jar containing 90% ethanol and incubate at RT for 5 min.
   vi. Immerse the sections in a glass staining jar containing 80% ethanol and incubate at RT for 5 min.
   vii. Immerse the sections in a glass staining jar containing nuclease-free water and incubate at RT for 15 min.
   viii. Remove glass slides from water, aspirate remaining water, and create a hydrophobic barrier around the section using PAP PEN.
   ix. Drop 50 μL of PBS onto the sections.
   x. Aspirate the PBS, drop 50 μL of 4% PFA in PBS onto the section and incubate at RT for 10 min.
   xi. Aspirate the PFA solution and wash the tissue sections twice with 50 μL of PBS.

Permeabilization

© Timing: 35–75 min

Permeabilization step is critical to enhancing the efficiency of in situ RT, as mentioned in “tissue preparation” of before you begin and Figure 1. Subsequent steps are performed on a laboratory bench suitable for molecular biological experiments.

2. Permeabilize sections with HCl (a) or TE (b) are as follows:
   a. HCl treatment (= 35 min).
      i. Aspirate PBS from the sections, drop 25 μL of 5% (v/v) Triton X-100 in PBS onto the section, and incubate at RT for 3 min.

   ▲ CRITICAL: The drop of 25 μL of Triton X-100 is the optimal volume to prevent leakage from a 1 × 1 cm hydrophobic barrier.

      ii. Aspirate Triton X-100 in PBS, wash the tissue sections twice with 50 μL of PBS.
      iii. Aspirate PBS, drop 50 μL of 0.1 N HCl in nuclease-free water on the section, and incubate at RT for 5 min.
      iv. Aspirate the HCl solution, drop 50 μL of 1 M Tris-HCl (pH 8.0) onto the section, and incubate at RT for 5 min.
v. Aspirate the Tris solution, immerse the sections in preheated PBS at 65°C in a polypropylene staining jar and incubate in a hybridization oven for 5 min to extend the RNAs.

vi. Immerse the sections in a polypropylene staining jar containing ice-cold PBS for at least 3 min until the sections are fully cooled.

vii. Place a paper towel on the bottom of the humidified chamber and add 40 mL of PBS to balance the vapor pressure (Figure 3A).

⚠ CRITICAL: Do not humidify with nuclease-free water. The vapor of the water will be absorbed by the higher salt solution loaded onto the sections, causing leakage from the hydrophobic barriers during incubation.

viii. Remove the glass slides from the PBS, aspirate remaining PBS, and place the glass slides in the humidified chamber.

ix. Wash the tissue sections twice with 50 μL of PBS.

b. TE treatment (∼75 min).

i. Aspirate the PBS from sections, immerse the sections in TE buffer (pH 8.0) preheated to 70°C in a polypropylene staining jar and incubate in a hybridization oven for 1 h.

ii. Immerse the sections in a polypropylene staining jar containing chilled PBS for at least 3 min until the sections are fully cooled.

iii. Place a paper towel on the bottom of the humidified chamber and add 40 mL of PBS to balance the vapor pressure.

iv. Remove the glass slides from the PBS, aspirate remaining PBS, and place the glass slides in the humidified chamber.

v. Wash the tissue sections twice with 50 μL of PBS (Figure 3B).

**In situ RT**

© Timing: approximately 1 h

mRNAs are reverse-transcribed in situ with NPOM-caged RT primers.

3. Prepare the NPOM-caged RT primer mix, shown in materials and equipment.

⚠ CRITICAL: NPOM is stable under indoor fluorescent light, but take care not to leave caged primers under the light for extended periods of time, unless required. By preparing RT primers with multiple barcodes, different samples can be pooled after cell lysis (step 26).

4. After spinning down the sample tube (1,400 × g, RT, 2 s), incubate the tubes at 65°C in a preheated thermal cycler for 5 min to relax the secondary structure of the primers and immediately place the tubes on ice for at least 2 min.

5. Prepare the first strand mix, as shown in materials and equipment.

6. Mix 6 μL of NPOM-caged RT primer mix (step 4) and 4 μL of first strand mix (step 5) in a 0.2 mL 8-strip PCR tube by gentle pipetting.

7. Add 10 μL of the NPOM-caged RT primer and the first strand mix (step 6) onto the section and seal with a round coverslip (φ15 mm) (Figure 3C).

⚠ CRITICAL: Ensure there are no air bubbles present on the section. The size of the coverslip can be changed according to the size of the section, with an equivalent size being the best option.

8. Place the sections in a PBS humidified chamber, and incubate at 42°C for 1 h in a hybridization oven (Figure 3D).

9. Drop 300 μL of PBS onto the coverslip and remove the coverslips using tweezers (Figure 3E).
CRITICAL: Be careful not to damage or peel tissue samples. The coverslips will float by dropping PBS onto the glass slides.

10. Immerse sections in PBS preheated to 70°C in a polypropylene staining jar and incubate in a hybridization oven for 10 min to terminate RT reaction.

11. Immerse the sections in a polypropylene staining jar containing chilled PBS for at least 3 min until the sections are fully cooled.

Pause point: Sections can be stored at 4°C for at least 3 days under light-shielded conditions.

12. Remove the glass slides from PBS, aspirate the remaining PBS, and place the glass slides in a humidified chamber.

13. Wash the tissue sections twice with 50 ℓL of PBS.

(Optional) Immunostaining

@ Timing: 3.5–24 h

Immunostaining is useful to visualize ROIs for subsequent photo-irradiation.

CRITICAL: Immunostaining protocols can largely depend on those recommended by antibody suppliers, but antigen retrieval by heating and proteinase treatment should be avoided. Overnight incubation at 4°C with primary antibodies is less harmful to the yield of sequence libraries. Secondary antibodies excited by blue (λ=488 nm), green (λ=555 nm), and far-red (λ=633 nm) light can be used for immunolocalization, but those excited by shorter wavelengths (<425 nm) are not compatible with PIC because their excitation uncages the NPOM moieties of the RT primers.

14. Aspirate PBS from the sections, add 50 μL of blocking solution, and incubate at RT for 15 min in a humidified chamber.

15. Aspirate the blocking solution, drop 50 μL of primary antibodies diluted in blocking solution onto the section, and incubate at RT for 2 h in a humidified chamber.

16. Aspirate the primary antibody solution, wash the sections three times with 50 μL of TBST.

17. Drop 50 μL of secondary antibodies diluted in blocking solution onto the section and incubate at RT for 1 h in a humidified chamber.

18. Aspirate the secondary antibody solution and wash the sections three times with 50 μL of TBST.

(Optional) Nuclear staining

@ Timing: approximately 20 min

Nuclear Violet is useful to observe nuclei with 426–450 nm excitation light without uncaging NPOM moieties. Nuclear Violet is also excited by ~365 nm UV, thus acting as a useful indicator of UV-irradiated cells.

19. Aspirate the TBST from the sections, add 50 μL of Nuclear Violet LCS1 (1:500) dissolved in blocking solution, and incubate at RT for 15 min in a humidified chamber.

CRITICAL: Nuclear staining can be performed simultaneously during incubation with the secondary antibody (step 17).

20. Aspirate the nuclear staining solution and wash the sections three times with 50 μL of TBST.
UV irradiation

Timing: approximately 5 min

NPOM moieties are uncaged by UV irradiation.

21. Aspirate the TBST from the sections and mount a round coverslip (φ15 mm) with 10 µL of SlowFade Diamond.

△ CRITICAL: Ensure that no air bubbles are present in the section. The size of the coverslip can be changed according to the size of the section, with an equivalent size being the best option.

22. Irradiate 365 nm centered UV light onto the ROIs for 3–15 min with DMD.

△ CRITICAL: We used a Leica DM6B fluorescence microscope equipped with Polygon1000-G DMD (Mightex) and UHP-F-365 LED (Prizmatix) for the photo-irradiation (Figure 4A). First, take a fluorescent image of the nuclear staining, with immunofluorescence as optional, using Leica Application Suite X (LAS-X) software, where the excitation wavelength must be longer than 425 nm to avoid uncaging of NPOM moieties. Next, overlay a PolyScan window, software to regulate the DMD, and outline the ROIs. Then, turn on the LED light to irradiate the ROI and obtain an image of the excited nuclei to record the illuminated area. When conducting the PIC experiment for the first time, it is recommended to also prepare a non-irradiated sample and to confirm that almost no product is obtained in the subsequent library synthesis (Figure 5), which is useful to check whether unexpected uncaging reactions are prevented.

Cell lysis

Timing: approximately 40 min

Whole cell lysates are collected in the test tubes.

23. Drop 300 µL of PBS onto the coverslip and remove coverslips using tweezers (Figure 3E).

△ CRITICAL: Be careful not to damage or peel tissue samples. The coverslips will float by dropping PBS onto the glass slides.

24. Wash the tissue sections twice with 50 µL of PBS.
25. Drop 40 µL of protease K lysis solution onto the sections and incubate for 15 min at 55°C in a humidified chamber in a hybridization oven.
26. Scrape the tissues and collect the cell lysates and debris in a 1.5 mL tube.

Note: Differentially barcoded samples can be pooled in the same tube.

27. Drop 40 µL of protease K lysis solution onto the sections and incubate for 15 min at 55°C in a humidified chamber in a hybridization oven.
28. Collect the cell lysates and debris in the same tube as step 26 (total volume ≈ 80 μL).
29. Vortex and spin down (1,400 × g, RT, 2 s).
30. Incubate for 15 min at 55°C in a hybridization oven.
31. Vortex and spin down (1,400 × g, RT, 2 s).
32. Incubate for 15 min at 55°C in a hybridization oven.
33. Vortex and spin down (1,400 × g, RT, 2 s).

Pause point: The samples can be stored at −20°C for at least 1 week under light-shielded conditions.

Purification of cDNA:mRNA hybrids

© Timing: approximately 10 min

cDNA:mRNA hybrids are purified by spin column.

34. Add 5 volumes of buffer PB from Qiagen MinElute PCR Purification kit (≈ 400 μL) to 1 volume of the sample (≈ 80 μL).
35. Vortex and spin down (1,400 × g, RT, 2 s).
36. Apply the lysates to MinElute Spin Column (Max 750 μL).
37. Centrifuge at 15,300 × g at RT for 1 min and discard flow through.
38. Add 750 μL of buffer PE to MinElute Spin Column.
39. Centrifuge at 15,300 × g at RT for 1 min and discard flow through.
40. Centrifuge at 15,300 × g at RT for 2 min and discard flow through.
41. Transfer the MinElute Spin Column to a new 1.5 mL tube.
42. Add 17 μL of nuclease-free water to the MinElute Spin Column and incubate at RT for 1 min.
43. Centrifuge at 15,300 × g at RT for 2 min and discard flow through.

Pause point: The samples can be stored at −20°C for at least 1 month under light-shielded conditions.

Second-strand DNA synthesis

© Timing: approximately 2.5 h
Second-strand DNA is synthesized using the nick translation method using RNase H, DNA polymerase I, and DNA ligase mix, by which only the cDNAs derived from the photo-irradiated region are filled in to the 5’ end of the T7 promoter.

44. Prepare second-strand mix, as shown in materials and equipment.
45. Mix 5 μL of second-strand mix (step 44) with 15 μL of purified cDNA:mRNA hybrids (step 43) in a 0.2 mL 8-strip PCR tube by gentle pipetting.
46. After spinning down the sample tube (1,400 × g, RT, 2 s), incubate the tubes at 16°C in a thermal cycler for 2 h and immediately place the tubes on ice.
47. Mix Ampure Beads and Beads Binding Buffer in a ratio of 1:5 and vortex thoroughly.
48. Add 24 μL of Ampure Beads and Beads Binding Buffer mix (step 47) to the second-stranded DNA sample (step 46) and vortex thoroughly.

△ CRITICAL: The volume of the Ampure Beads and Beads Binding Buffer mix (step 47) to be added is 1.2 times the volume of the second-stranded DNA sample (step 46). Different bar-coded samples can be pooled in the same tube in this step.

49. After spinning down the sample tube (1,400 × g, RT, 2 s), incubate the tubes at RT for 15 min.
50. Place the sample tube on a magnetic stand and wait for at least 5 min.

△ CRITICAL: The waiting time depends on the performance of the magnetic stand. Make sure the beads are fully bonded to the magnet of the stand.

51. Remove the supernatant.

△ CRITICAL: Be careful not to aspirate the beads.
52. Add 200 μL of 80% ethanol and incubate at RT for 30 s.
53. Remove the supernatant.
54. Add 200 μL of 80% ethanol and incubate at RT for 30 s.
55. Remove the supernatant and spin down the sample tube (1,400 × g, RT, 2 s).
56. Place the sample tube on the magnetic stand and incubate at RT for 1 min.
57. Remove the supernatant and air dry for 2 min.

△ CRITICAL: Ensure that no ethanol remains in the tube.
58. Suspend the beads with 6.4 μL of nuclease-free water by pipetting and incubate at RT for at least 2 min in the non-magnetic stand.

IVT

Θ Timing: 15–17 h (overnight reaction)
cDNAs derived from photo-irradiated regions are linearly amplified as RNAs (aRNAs) via IVT reaction.

59. Prepare IVT mix, as shown in materials and equipment.
60. Add 9.6 μL of IVT mix (step 59) to the bead suspension containing second-stranded cDNA (step 58) in a 0.2 mL 8-strip PCR tube by gentle pipetting.
61. After spinning down the sample tube (1,400 × g, RT, 2 s), incubate the tubes at 37°C in a thermal cycler for 15–17 h and immediately place the tubes on ice.
**Purification of aRNA**

© Timing: approximately 1 h

62. Add 1 μL of TURBO DNase from the MEGAscript T7 Transcription Kit.
63. After tapping and spinning down the sample tube (1,400 × g, RT, 2 s), incubate the tubes at 37°C in a thermal cycler for 15 min and immediately place the tubes on ice.
64. Add 3 μL of ExoSAP-IT Express PCR Product Cleanup.
65. After tapping and spinning down the sample tube (1,400 × g, RT, 2 s), incubate the tubes at 37°C in a thermal cycler for 5 min and immediately place the tubes on ice.
66. Add 5.5 μL of Fragmentation Buffer and vortex thoroughly.
67. After spinning down the sample tube (1,400 × g, RT, 2 s), incubate the tubes at 94°C in a pre-heated thermal cycler for 3 min and immediately place the tubes on ice.
68. Add 2.75 μL of 0.5 mol/L EDTA Solution (pH 8.0) and vortex thoroughly.
69. After spinning down the sample tube (1,400 × g, RT, 2 s), place the sample tube on the magnetic stand and incubate at RT for 5 min.
70. Collect 28 μL of supernatant in a new 0.2 mL 8-strip PCR tube.
71. Add 50.4 μL of RNAClean XP beads and vortex thoroughly.

△ CRITICAL: The volume of RNAClean XP beads to be added is 1.8 times the volume of the sample.

72. After spinning down the sample tube (1,400 × g, RT, 2 s), incubate at RT for 10 min.
73. Place the sample tube on a magnetic stand and wait for at least 5 min.

△ CRITICAL: The waiting time depends on the performance of the magnetic stand. Make sure the beads are fully bonded to the magnet of the stand.

74. Remove the supernatant.

△ CRITICAL: Be careful not to aspirate the beads.

75. Add 200 μL of 70% ethanol and incubate at RT for 30 s.
76. Remove the supernatant.
77. Add 200 μL of 70% ethanol and incubate at RT for 30 s.
78. Remove the supernatant and spin down the sample tube (1,400 × g, RT, 2 s).
79. Place the sample tube on the magnetic stand and incubate at RT for 1 min.
80. Remove the supernatant and air dry for 2 min.

△ CRITICAL: Ensure that no ethanol remains in the tube.

81. Resuspend the beads with 6.5 μL of nuclease-free water by pipetting and incubate at RT for at least 2 min.
82. After spinning down the sample tube (1,400 × g, RT, 2 s), place the sample tube on the magnetic stand and incubate at RT for 5 min.
83. Collect 4.5 μL of supernatant in a new 0.2 mL 8-strip PCR tube.

‖ Pause point: The samples can be stored at −80°C for at least 1 month after IVT.

**(Optional) Confirmation of aRNA by gel electrophoresis**

© Timing: approximately 1 h
84. The size and amount of aRNA can be confirmed using gel electrophoresis.

**Reverse transcription of aRNAs**

⏱ Timing: approximately 1.5 h

aRNAs are further reverse-transcribed by random primers with the Illumina Read2 sequence.

85. Prepare aRNA–N6 primer mix, as shown in materials and equipment.
86. Spin down the sample tube (1,400 × g, RT, 2 s).
87. Incubate the tubes at 65°C in a preheated thermal cycler for 5 min to relax the secondary structure of the aRNAs and immediately place the tubes on ice.
88. Prepare the RT mix, as shown in materials and equipment.
89. Add 4 μL of RT mix (step 88) to the aRNA–N6 primer mix (step 87) and mix by gentle pipetting. After spinning down the sample tube (1,400 × g, RT, 2 s), incubate at 25°C for 10 min, and then at 42°C for 1 h in a thermal cycler.

⚠️ Pause point: The samples can be stored at 4°C for at least 1 day.

**PCR amplification**

⏱ Timing: approximately 1 h

Sequence libraries are amplified by 11 cycles of PCR.

⚠️ CRITICAL: The PCR cycles can be increased by 15 if the library yield is expected to be low due to the small number of target cells. More cycles of PCR will result in UMI saturation by over-amplification.

90. Prepare the library PCR mix, as shown in materials and equipment.
91. After spinning the sample tube (1,400 × g, RT, 2 s), amplify the samples using PCR, as shown below.

| Steps                | Temperature | Time  | Cycles |
|----------------------|-------------|-------|--------|
| Initial Denaturation | 98°C        | 30 s  | 1      |
| Denaturation         | 98°C        | 10 s  | 11 cycles |
| Annealing            | 60°C        | 30 s  |        |
| Extension            | 72°C        | 30 s  |        |
| Final extension      | 72°C        | 10 min| 1      |
| Hold                 | 4°C         | forever|        |

⚠️ Pause point: The samples can be stored at −20°C for at least 1 month.

92. Add 1 μL of RNase A (10 mg/mL).
93. After tapping and spinning down the sample tube (1,400 × g, RT, 2 s), incubate the tubes at 37°C in a thermal cycler for 30 min.

**Size selection of sequence libraries (1st round)**

⏱ Timing: approximately 1 h

⚠️ CRITICAL: At least two rounds of size selection are recommended. Perform more rounds if the sizes do not fall within 200–400 bp.
94. Add 6 μL of nuclease-free water to the PCR products and adjust to 50 μL.
95. Mix 50 μL of PCR products with 32.5 μL of Ampure XP beads and vortex thoroughly.
96. After spinning down the sample tube (1,400 × g, RT, 2 s), incubate at RT for 15 min.
97. Place the sample tube on a magnetic stand and wait for at least 5 min.

△ CRITICAL: The waiting time depends on the performance of the magnetic stand. Make sure the beads are fully bonded to the magnet of the stand.

98. Collect the supernatant in a new 0.2 mL 8-strip PCR tube.
99. Add 12.5 μL Ampure XP beads, and vortex thoroughly.
100. After spinning down the sample tube (1,400 × g, RT, 2 s), incubate at RT for 10 min.
101. Place the sample tube on a magnetic stand and wait for at least 5 min.

△ CRITICAL: The waiting time depends on the performance of the magnetic stand. Make sure the beads are fully bonded to the magnet of the stand.

102. Remove the supernatant.

△ CRITICAL: Be careful not to aspirate the beads.

103. Add 200 μL of 80% ethanol and incubate at RT for 30 s.
104. Remove the supernatant.
105. Add 200 μL of 80% ethanol and incubate at RT for 30 s.
106. Remove the supernatant and spin down the sample tube (1,400 × g, RT, 2 s).
107. Place the sample tube on the magnetic stand and incubate at RT for 1 min.
108. Remove the supernatant and air dry for 2 min.

△ CRITICAL: Ensure that no ethanol remains in the tube.

109. Suspend the beads with 32 μL of nuclease-free water by pipetting and incubate at RT for at least 2 min.
110. After spinning down the sample tube (1,400 × g, RT, 2 s), place the sample tube on the magnetic stand and incubate at RT for 5 min.
111. Collect 30 μL of supernatant in a new 0.2 mL 8-strip PCR tube.

◆◆ Pause point: The samples can be stored at −20 °C for at least 6 months.

Size selection of sequence libraries (2nd round)

☉ Timing: approximately 1 h

112. Mix 30 μL of the sample (step 117) with 19.5 μL of Ampure XP beads and thoroughly vortex.
113. After spinning down the sample tube (1,400 × g, RT, 2 s), incubate at RT for 15 min.
114. Place the sample tube on a magnetic stand and wait for at least 5 min.

△ CRITICAL: The waiting time depends on the performance of the magnetic stand. Make sure the beads are fully bonded to the magnet of the stand.

115. Collect the supernatant in a new 0.2 mL 8-strip PCR tube.
116. Add 7.5 μL Ampure XP beads and vortex thoroughly.
117. After spinning down the sample tube (1,400 × g, RT, 2 s), incubate at RT for 10 min.
118. Place the sample tube on a magnetic stand and wait for at least 5 min.
△ CRITICAL: The waiting time depends on the performance of the magnetic stand. Make sure the beads are fully bonded to the magnet of the stand.

119. Remove the supernatant.

△ CRITICAL: Be careful not to aspirate the beads.

120. Add 200 μL of 80% ethanol and incubate at RT for 30 s.
121. Remove the supernatant.
122. Add 200 μL of 80% ethanol and incubate at RT for 30 s.
123. Remove the supernatant and spin down the sample tube (1,400 × g, RT, 2 s).
124. Place the sample tube on the magnetic stand and incubate at RT for 1 min.
125. Remove the supernatant and air dry for 2 min.

△ CRITICAL: Ensure that no ethanol remains in the tube.

126. Suspend the beads with 15 μL of nuclease-free water by pipetting and incubate at RT for at least 2 min.
127. After spinning down the sample tube (1,400 × g, RT, 2 s), place the sample tube on the magnetic stand and incubate at RT for 5 min.
128. Collect 12 μL of supernatant in a new 0.2 mL 8-strip PCR tube.

Pause point: The samples can be stored at −20°C for at least 6 months.

Quantification of the library

© Timing: 1–2 h

129. Measure the size distribution of the PIC library using a bioanalyzer by loading 1 μL on a chip in a High Sensitivity DNA Kit, according to the manufacturer’s instructions.

△ CRITICAL: The expected size is 200–400 bp (Figure 5), otherwise repeat the size selection (steps 100–134).

Sequencing and data analysis

© Timing: ≥ 1 day

130. Perform paired-end sequencing on an Illumina sequencer (for example, HiSeq or NovaSeq) following the manufacturer’s instructions. We usually add PhiX at 5% for the final spike in and take 10–20 million reads (Read1, 12 cycles for UMI and barcode 18; Read2, ≥ 50 cycles for cDNA), which may vary depending on the ROI area and library yield.
131. Extract the barcodes and UMIs in the reads using UMI-tools with the following command:

```bash
umi_tools extract -I read1.fastq -read2-in=read2.fastq -bc-pattern=NNNNNNCCCCCC -read2-stdout
```

132. Trim the adapter sequence from the reads using Trim Galore! (version 0.6.6) with the following command:
133. Map the reads by aligning the HISAT2 software to the appropriate reference genome (GRCm38, in this case).

134. Determine the read counts per gene with featureCounts and UMI-tools with the following command:

```bash
featureCounts -a GRCm38.gtf -R BAM input.bam
samtools index input.featureCounts.bam
umi_tools count --method=unique --per-gene --per-cell --gene-tag=XT -I input.featureCounts.bam
```

135. Using the resulting counts, extract differentially expressed genes (DEGs) using the R library DESeq2, which is also used to transform the count data into regularized log data before performing principal component analysis (PCA) using the R prcomp function.

**EXPECTED OUTCOMES**

In the present experiment, we prepared fresh-frozen sections and formalin-fixed frozen and paraffin sections from the adult mouse hippocampus. HCl or TE was adopted in the permeabilization step for the fresh-frozen sections, whereas TE alone was used for the other sections. After performing in situ RT with NPOM-caged RT primer, CA1, CA3, or DG regions of the hippocampus were photo-irradiated with DMD (Figure 4A; n = 4 biological replicates for each condition). After the library synthesis, BioAnalyzer analysis showed that the size of the library was centered to 250 bp, and fresh-frozen sections yielded an average of 10.4 and 15.8 nM for HCl or TE treatment, respectively, in the final volume of 12 μL (Figure 4B). However, the yields of formalin-fixed tissues were lower, 2.4 nM for frozen sections and 0.57 nM for paraffin sections. Sequencing results showed that all samples provided approximately 1 × 10⁶ reads, and about half of them in the frozen sections were assigned to the genes, as indicated in a benchmark paper for CEL-seq2 (Mereu et al., 2020), while slightly fewer were assigned for the paraffin sections (Figure 4B). The number of gene-assigned UMIs and the genes detected were better in unfixed tissues (averaging 6.5 × 10⁵ UMIs / 17,392 genes and 6.5 × 10⁵ UMIs / 16,186 genes in HCl or TE-permeabilized tissues, respectively) relative to formalin-fixed frozen or paraffin sections (1.4 × 10⁵ UMIs / 10,190 genes or 8.9 × 10⁴ UMIs / 10,548 genes, respectively; Figure 4C). The UMI count data were normalized with DESeq2 and subjected to PCA using prcomp in the R package, in which all samples were separated into three groups according to the photo-irradiated regions (CA1, CA3, and DG; Figure 4D). DEG analysis was conducted with DEseq2 by comparing CA1 and DG in each condition and showed that known marker genes (Cembrowski et al., 2016) were detected as DEGs in the frozen sections despite fixation (Figure 4E; Wfs1 [CA1 marker], Ociad2 and Dkk3 [CA1 and CA3 markers], Prox1, and Pdzd2 [DG markers]). In contrast, in paraffin sections, genes with high average expression (Dkk3 and Prox1) were detected as statistically significant DEGs, whereas genes with low average expression (Wfs1, Ociad2, and Pdzd2) were not, but the fold changes were all oriented toward the expected direction (Figure 4E). These results indicate PIC can isolate transcriptome information from the photo-irradiated region not only in fresh-frozen sections but also in formalin-fixed frozen or paraffin sections. Because the formalin-fixed samples were inferior to fresh-frozen samples in terms of the number of detected genes and the detection sensitivity of DEGs, it is expected these will provide better performance by sequencing a larger number of reads and by preparing more biological replicates for the experiment.

**LIMITATIONS**

In the PIC method, multiple ROIs can be irradiated simultaneously, but they cannot be separated as different samples. A total of 3 × 10⁶ reads is often sufficient for sequencing analysis, but 1–2 × 10⁷
reads are recommended for small ROIs and/or for comparison between ROIs with similar characteristics. Empirically, a transcriptome of sufficient depth can be obtained with over 100 cells, but we have experienced that library yields are much lower in some tissues, such as the myocardium and aorta. Therefore, it is necessary to consider the number of cells to be irradiated for the tissues being analyzed for the first time. The first step was to irradiate 100 cells and monitor the library synthesis using a BioAnalyzer. If the library yield is lower than desired, the number of irradiated cells should be increased.

**TROUBLESHOOTING**

**Problem 1**
No/reduced immunostaining (step 22).

**Potential solution**
Loss of antigens: Increase the fixation time to prevent dissipative loss of antigens (see Figure 1).

Poor antibody specificity: Test with an antibody that does not require antigen retrievals or is reactive to the antigens even after permeabilization with HCl or TE-heating.

**Problem 2**
No/reduced library yield.

**Potential solution**
Degraded RNA: Frozen tissues should be stored at $-80°C$, but store paraffin blocks at $4°C$ or lower. After sectioning, proceed with PIC experiment immediately or store the sections at $-80°C$.

Over-fixation: Reduce the fixation time or use fresh-frozen tissues to decrease the cross-linkage of mRNAs. Empirically, even fixation for 10 min improves preservation of tissue morphology of small organs and embryos, and fixation for 6 h effectively prevents dissipative antigen loss (Figure 1B).

Low cell number for ROIs: Try to irradiate 100 or more cells (step 22). In addition, barcoded in situ RT (step 3) and pooling (step 26) of multiple samples can reduce the loss in subsequent library synthesis.

Insufficient uncaging: Maximize the power of the light source and increase photo-irradiation time (step 22; Figure 2F).

**Problem 3**
Background signal from non-irradiated samples.

**Potential solution**
Unexpected uncaging: Order an OPC-grade purification rather than an HPLC purification (see “synthesis of caged RT primer” in before you begin). Ensure that the samples are shielded from light when storing the caged primer and conducting the extended period incubation steps, such as in situ RT, antibody reactions, cell lysis, and second-strand DNA synthesis (steps 8, 14, 15, 17, 19, 25, 27, 30, and 32).

**Problem 4**
Decreased detection of DEGs (step 135).

**Potential solution**
Low number of replicas: For each sample group, prepare at least 3–4 replicates and more, if possible, to increase statistical power.
Contamination by unexpected uncaging: Ensure that the samples are light shielded, as noted above. When observing the nuclear staining and immunostaining images, make sure to excite with ≥425 nm wavelength and ensure that the DMD light source is turned off.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Shinya Oki (oki.shinya.3w@kyoto-u.ac.jp).

Materials availability
All materials are available commercially.

Data and code availability
The datasets generated during our study are available on GEO with accession number GSE195459 and are referenced in the original study.

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AUTHOR CONTRIBUTIONS

Y.O. and S.O. conceived the PIC and supervised all aspects of the work. M.H., R.K., and A.H. performed the experiments. M.H., K.M., K.T., Y.O., and S.O. analyzed the sequencing data. M.H., Y.O., and S.O. wrote the manuscript. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS

S.O. and Y.O. are involved in a pending patent related to PIC technology (application number, 2019-094216; patent office, Japan Patent Office; current status, patent pending). All the other authors declare no competing interests.

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