Tissue-specific DamID protocol using nanopore sequencing

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ABSTRACT

DNA adenine methylation identification (DamID) is a powerful method to determine DNA binding profiles of proteins at a genomic scale. The method leverages the fusion between a protein of interest and the Dam methyltransferase of *E. coli*, which methylates proximal DNA *in vivo*. Here, we present an optimized procedure, which was developed for tissue-specific analyses in *Caenorhabditis elegans* and successfully used to footprint genes actively transcribed by RNA polymerases and to map transcription factor binding in gene regulatory regions. The present protocol details *C. elegans*-specific steps involved in the preparation of transgenic lines and genomic DNA samples, as well as broadly applicable steps for the DamID procedure, including the isolation of methylated DNA fragments, the preparation of multiplexed libraries, Nanopore sequencing, and data analysis. Two distinctive features of the approach are (i) the use of an efficient recombination-based strategy to selectively analyze rare cell types and (ii) the use of Nanopore sequencing, which streamlines the process. The method allows researchers to go from genomic DNA samples to sequencing results in less than a week, while being sensitive enough to report reliable DNA footprints in cell types as rare as 2 cells per animal.

Keywords: CRE recombinase-based, tissue-specific expression, DNA adenine methylation identification, DNA-protein interaction, long-read sequencing, third generation sequencing

BACKGROUND

The advent of deep sequencing has revolutionized basic and applied biomedical science. Analyzing the expression of an entire genome, how its expression is modified by intra- and extra-cellular factors, or obtaining the genomic footprints of DNA-binding proteins have become routine procedures in many research projects. However, when working with entire animals, evaluating genome-wide changes for a given cell type often remains challenging, as it requires to either sort cells from a dissociated tissue of interest or use transgenic approaches to purify transcripts from the target cell type [1-4]. This can be difficult when the considered cell type is hard to separate from surrounding cells or is a rare cell type. In particular, in the nematode *C. elegans* for which no continuous culture system for differentiated cells has been developed, this is a major hurdle to identify expressed genes or determine interaction profiles of DNA-binding proteins in rare cell types.

DNA adenine methylation identification

DNA adenine methylation identification (DamID) [5] was developed in 2000 by Bas van Steensel and Steven Henikoff as an alternative to chromatin immuno-precipitation (ChIP) to identify genome-wide DNA-protein interactions *in vivo*, avoiding the troublesome steps of cell or nucleus isolation, fixation and immunoprecipitation with antibodies. DamID relies on the expression of trace levels of the *E. coli* DNA adenine methyltransferase (Dam) fused to a protein of interest (POI). The Dam methylates DNA located close to the binding site of the POI at GATC sites (**Fig. 1A**). Methylated sites are then used to track POI binding sites. Notably, DamID is highly versatile and has been applied in many organisms, ranging from fission yeast [6-10], *C. elegans* [11-17], Drosophila [18-23], Medaka [24], Arabidopsis [25-27], and mice [28-31] to human cells [32-35]. Additionally, DamID has been adapted to evaluate an extensive range of chromatin features *in vivo* such as chromatin accessibility (CATaDA) [36], chromatin dynamics visualization (m6A-tracer) [37], transcription factor binding (MaTaDa)

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[38] and co-binding (SpDamID) [39], RNA-DNA interactions (RNA-DamID) [40], three-dimensional genome organization (DamC) [41], transcriptional profiling (TaDa [42] and RAPID [43]), or simultaneous transcription and protein-DNA interaction profiling in single cells (scDam&T-seq) [44,45].

Development of tissue-specific DamID in *C. elegans*

We recently developed RAPID (RNA PoI DamID) to profile genome-wide, tissue-specific RNA Polymerase occupancy *in vivo* without cell isolation or nuclei purification [43]. In contrast to other methods, which sequence transcripts from specific cell types, RAPID is based on the methylation footprinting by an RNA polymerase subunit fused to a Dam methyltransferase, expressed only in the target tissue (**Fig. 1B** and **1C**). Using DNA purified from entire animals, we profiled muscle and intestine transcription, with results comparable to other tissue-specific RNA-seq methods based on sorting (Fluorescence Activated Cell or Nuclei Sorting: FACS [1], FANS [2]) or biochemical purification (trans-splicing-based RNA tagging, SRT [3], and polyA tagging and sequencing, PAT-seq [4]). We further showed that this method can be applied for rare cell types by profiling cell types representing only two cells per animal or 0.2% of the somatic cells. The specific advantages and limitations of RAPID as compared to RNA-seq-based methods were extensively discussed in this previous work [43].
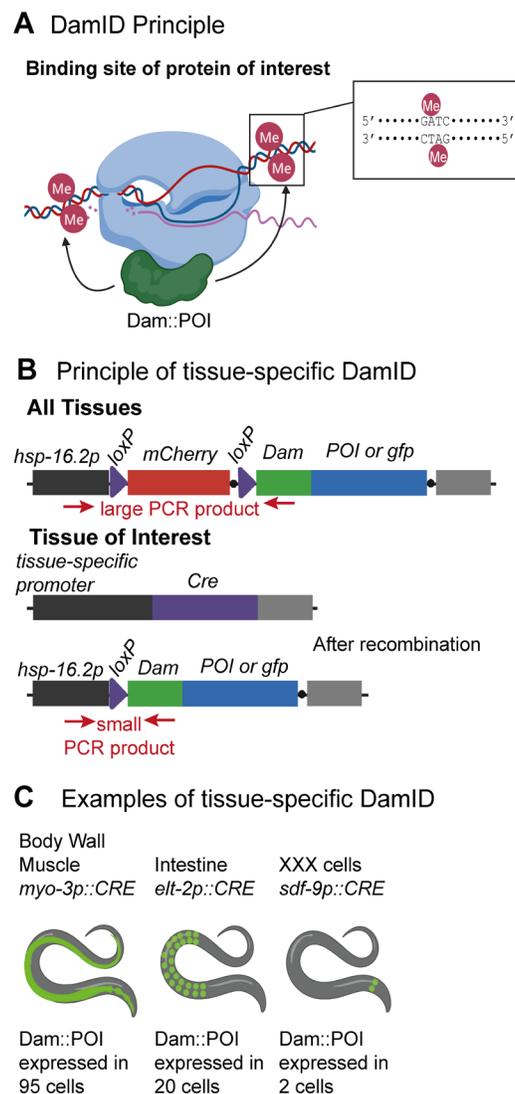
Figure 1. Tissue-specific DamID schemes. A. DamID is used to identify genome-wide DNA-protein interactions *in vivo*. DNA adenine methyltransferase (Dam) fused to a protein of interest (POI, here a subunit of the RNA polymerase) methylates GATC sites, forming unique flags to track genome regions where the POI was associated. **B.** Recombination-based transgenic approach for tissue-specific DamID. Tissue-specific DamID is achieved by *in vivo* CRE recombination in the tissue of interest, deleting a floxed *mCherry::STOP* cassette and enabling the transcription of the Dam::POI fusion or the Dam::GFP fusion, respectively. The later one is used to normalize for overall chromatin accessibility. Red arrows represent primers used to verify CRE recombination (see **Fig. 4**). **C.** Illustration of tissues already successfully used with tissue-specific DamID representing from 10% to 0.2% of the somatic cell content of the animal.

Although we applied this approach to RNA polymerase, the same approach can additionally be applied to profile the genome-wide interactions of any POI in a tissue-specific manner. Tissue-specific profiling requires only a modest number of animals (4000–5000), thereby reducing the time required to grow biological sample populations. Additionally, we coupled the methylation footprinting method with long-read sequencing technology using Oxford Nanopore technologies (ONT) [46]. This further reduces the data acquisition time, because sequencing can be performed in every laboratory without the requirement for a sequencing facility. Here, we present a bench protocol allowing one to perform methyl footprinting from DNA extraction to amplicon sequencing in less than a week.

Tissue-specific DamID in *C. elegans*

DamID requires the expression of a Dam fusion protein, which, if expressed at moderate to high levels, could in some instances lead to toxicity and potentially to mutations (data not shown) [42]. This is solved in *C. elegans* by the use of inducible promoters in the uninduced state to achieve very low expression levels. These promoters are however broadly expressed and therefore unadapted for tissue-specific profiling. In our tissue-specific DamID approach, the Dam-POI expression is driven by a heat shock promoter flanked in 5' by a loxP-mCherry-STOP-loxP cassette impairing its expression in non-target tissues (**Fig. 1B**). An additional construct drives the CRE recombinase expression in a cell-type specific manner, excising the STOP-cassette and thereby allowing basal expression of the Dam-POI. DamID requires a control for chromatin accessibility, as methylation by the POI Dam fusion depends on it. As a control for overall chromatin accessibility, we use a similar mCherry floxed GFP-Dam construct, which is expressed in the same tissue in a control line treated in parallel.

Critical aspects to consider before performing tissue-specific DamID

DamID has many advantages, including its very low background noise and exquisite sensitivity. However, before embarking on the use of tissue-specific DamID, it is essential to be aware of its limits.

Steric effects of Dam fusion: One of the initial points to consider is whether the Dam should be fused at the N- or C-terminus of the POI, in order to minimize the steric effect of the Dam and its impact on POI functionality, including its DNA-binding ability. This choice could be potentially guided by pre-existing information on the functional or binding domains of the POI, or by data from previous experiments in which fusion protein constructs have been functionally evaluated. Even if a systematic comparison of N- and C-terminal fusion designs on different transcription factors has shown robust target footprints regardless of the Dam position, the potential Dam steric effect remains hard to predict and may vary on a case-by-case basis [47].

Spatial resolution: DamID spatial resolution depends on the density of GATC sites in the genome. *C. elegans* has 269049 GATC motifs per haploid genome [48], corresponding to a mean distance between sites of 374 bp and a median of 210 bp [49]. With this density, DamID was successfully applied to obtain the genomic footprint of several transcription factors such as DAF-16 [13], CEH-60 [14] and CRH-1 (our unpublished data), as well as RNA Polymerases [43]. However, for most genomic regions the binding sites are defined as relatively large windows of several hundred bases, sometimes much larger in some regions with low GATC density. This corresponds to a spatial resolution substantially lower than a typical interval determined by ChIP.

Methylation dynamics: Understanding the methylation dynamics is essential to plan and interpret DamID experiments. Three aspects are particularly important. First, the irreversibility of DNA methylation in *C. elegans*, which lacks any relevant DNA demethylase activity. In a non-replicating *C. elegans* cell, methylation marks will therefore be irreversibly deposited. Second, the impact of DNA replication. Methyl marks will stay until DNA replication occurs and one DNA strand is passively demethylated through the DNA replication process. Hemimethylated DNA is not cut by DpnI and will remain undetected. Third, considering cell populations, the average methylation at a given site will build up over time. If Dam-POI interactions are transient or intermittent the level of methylation is expected to increase more slowly than for robust, long-lasting interactions. Consistent with these theoretical considerations, the profiles obtained in post-mitotic cells of adult worms tend to be cleaner and with better correlation between replicates compared to those obtained in embryos [11,43], presumably because the Dam-POI fusion protein has been expressed for a very short time in the early stages of development with fast-replicating cells. Eventually, a good knowledge about the timing of the cell lineage replication and the CRE transgene expression onset is essential to plan DamID experiments and interpret their results.

MATERIALS

Construction of DamID tissue-specific strains

- ✓ Plasmid #506 [*hsp-16.2p::loxP::mCherry::STOP::loxP::degron::dam::rpb-6::unc-54 3'UTR*]
- ✓ Plasmid #418 [*hsp-16.2p::loxP::mCherry::STOP::loxP::degron::gfp::dam::unc-54 3'UTR*]
- ✓ Plasmid pSR33 [*hsp-16.2::egl-13nls::CRE::tbb-2 3'UTR*]
- ✓ *C. elegans* gDNA or vector bearing the gene or promoter of interest.
- ✓ *Pac*I restriction enzyme (R0547S, NEB)
- ✓ *Kpn*I restriction enzyme (R3142S, NEB)
- ✓ *Nru*I restriction enzyme (R3192S, NEB)
- ✓ *Not*I restriction enzyme (R3189S, NEB)
- ✓ Standard molecular biology reagents and materials of high analytic grade for cloning (primers to amplify the gene of interest, primers to amplify the promoter of interest, high-fidelity polymerase, PCR-tubes, filter tips, micropipettes, LB-Amp plates)
- ✓ Thermocycler
- ✓ Gibson Assembly® Master Mix (E2611S, NEB)
- ✓ NEB® 5-alpha Electrocompetent *E. coli* (C2989K, NEB)
- ✓ Primer *Dam*-forward (5'-GAACCGCTACGGTTACAACGG)
- ✓ Primer *unc*-54-reverse (5'-TAGGGGGTGGGAGCACAG)
- ✓ Primer M13 (-21) forward (5'-TGTAAAACGACGGCCAG)
- ✓ Primer M13 (-40) reverse (5'-CAGGAAACAGCTATGACC)
- ✓ Standard materials and equipment for DNA agarose gel electrophoresis
- ✓ Materials required for Mos1-mediated Single-Copy Integration (MosSCI), including microinjection equipment, nematode host strains, co-injection plasmids [50].
- ✓ Reporter line SV1361 [*rps-27p::loxP::NLS::mCherry::let858 UTR::loxP::NLS::GFP::let-858 UTR*]

Nematode culture

- ✓ NGM plates (100 mm)
- ✓ GM48 or GM119 (Dam-) *E. coli* available from https://www.dsmz.de/ or https://cgsc.biology.yale.edu/
- ✓ M9 buffer
- ✓ Tween 20
- ✓ 13% w/v Sodium hypochlorite solution
- ✓ 5 M KOH
- ✓ 15 ml falcon tubes
- ✓ 1.5 ml microcentrifuge tubes
- ✓ 15 ml serological pipettes
- ✓ Glass Pasteur pipettes
- ✓ P20, P200 and P1000 µl pipettes
- ✓ 20, 200 and 1000 µl pipettes tips
- ✓ Swinging bucket centrifuge (*e.g.*, eppendorf microcentrifuge 5804 R)
- ✓ Fixed-Angle centrifuge (*e.g.*, eppendorf centrifuge 5415 D)

Purification of gDNA

- ✓ DNeasy Blood and Tissue Kit (69504, QIAGEN)
- ✓ RNase A (19101, QIAGEN)
- ✓ Ammonium acetate 3 M
- ✓ Ethanol (Molecular biology grade)
- ✓ Heating block with shaking function (*e.g.*, eppendorf ThermoMixerC)
- ✓ Swinging bucket centrifuge (*e.g.*, eppendorf microcentrifuge 5804 R)

Digestion and purification of methylated DNA

- ✓ *Dpn*I restriction enzyme (R0176L, NEB)
- ✓ Thermocycler (*e.g.*, Labcycler 48 Gradient-Thermal Cycler, 013-101, Witec AG)
- ✓ Primers
  - • double-stranded adapters (ds-adapters)
  - • AdRt (5′–CTAATACGACTCACTATAGGGCAGCGTG–GTCGCGGCCGAGGA)
  - • AdRb (5′–TCCTCGGCCG)
  - • PCR primer
  - • Adr (5′–NNNNGGTCGCGGCCGAGGATC; 50 µM)
- ✓ T4 DNA LIGASE (EL0013/10548730, Thermo Fisher Scientific)
- ✓ dNTPs PCR grade 4 × 25 µmol (11969064001, Sigma/Roche)
- ✓ HiDi DNA polymerase (myPOLS#9001 M)
- ✓ Standard molecular biology reagents and materials of high analytic grade for digestion and PCR (0.2 ml PCR-tubes, filter tips, micropipettes, 1.5 ml tubes)
- ✓ Agencourt AMPure XP (A63880, Beckman Coulter, Inc.; or compatibles beads)
- ✓ Magnetic particle rack for 1.5 ml tubes (*e.g.*, 12321D, Thermo Fisher Scientific or Z5342, Promega)
- ✓ Qubit dsDNA BR Assay Kit (10146592, Thermo Fisher Scientific)
- ✓ Tube Qubit assay thin-walled (12037609, Thermo Fisher Scientific)
- ✓ Standard materials and equipment for DNA agarose gel electrophoresis

Multiplexed library preparation for DamID nanopore sequencing

- ✓ NEBNext Ultra II End-Repair/dA-Tailing Module (E7546S, NEB)

NOTE: This module is part of the Ultra™ II workflow and is compatible for library construction in both Illumina® and Oxford Nanopore Technologies® workflows.

- ✓ Ligation Sequencing kit (SQK-LSK109, ONT)
- ✓ Native Barcoding Expansion kit (EXP-NBD104 [1-12] or EXP-NBD114 [13-24], ONT)

NOTE: Native Barcoding Expansion, is a kit to multiplex samples using PCR-free method to preserve additional information such as base modifications. Using both expansion kits (EXP-NBD104 and EXP-NBD114), there are 24 unique barcodes, allowing the user to pool up to 24 different samples in one sequencing experiment.

- ✓ Flow Cell Priming kit (EXP-FLP001, ONT)
- ✓ NEB Blunt/TA Master Mix (M0367S, NEB)
- ✓ NEBNext Quick Ligation Module (E6056S, NEB)
- ✓ AMPure XP 1.8 beads (A63880, Agencourt)
- ✓ Magnetic particle rack for PCR tubes (*e.g.*, 492025, Thermo Fisher Scientific or S1515S, NEB)
- ✓ Low Protein Binding (LoBind) Tubes (90410, Thermo Fisher Scientific)
- ✓ 0.2 ml UltraFlux SnapStrip PCR Tubes With Clear Flat Caps (LC384, Labgene Scientific)
- ✓ Biosphere Quality Tips (70.1114.200, Biosphere)
- ✓ Nanopore flow Cell (FLO-MIN106D, ONT)
- ✓ Thermocycler (*e.g.*, Labcycler 48 Gradient-Thermal Cycler, 013-101, Witec AG)
- ✓ Standard molecular biology reagents and materials of high analytic grade for library preparation (PCR-tubes, filter tips, micropipettes, 1.5 ml tubes)

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PROCEDURE AND ANTICIPATED RESULTS

The protocol presented below is visually summarized in **Figure 2**.
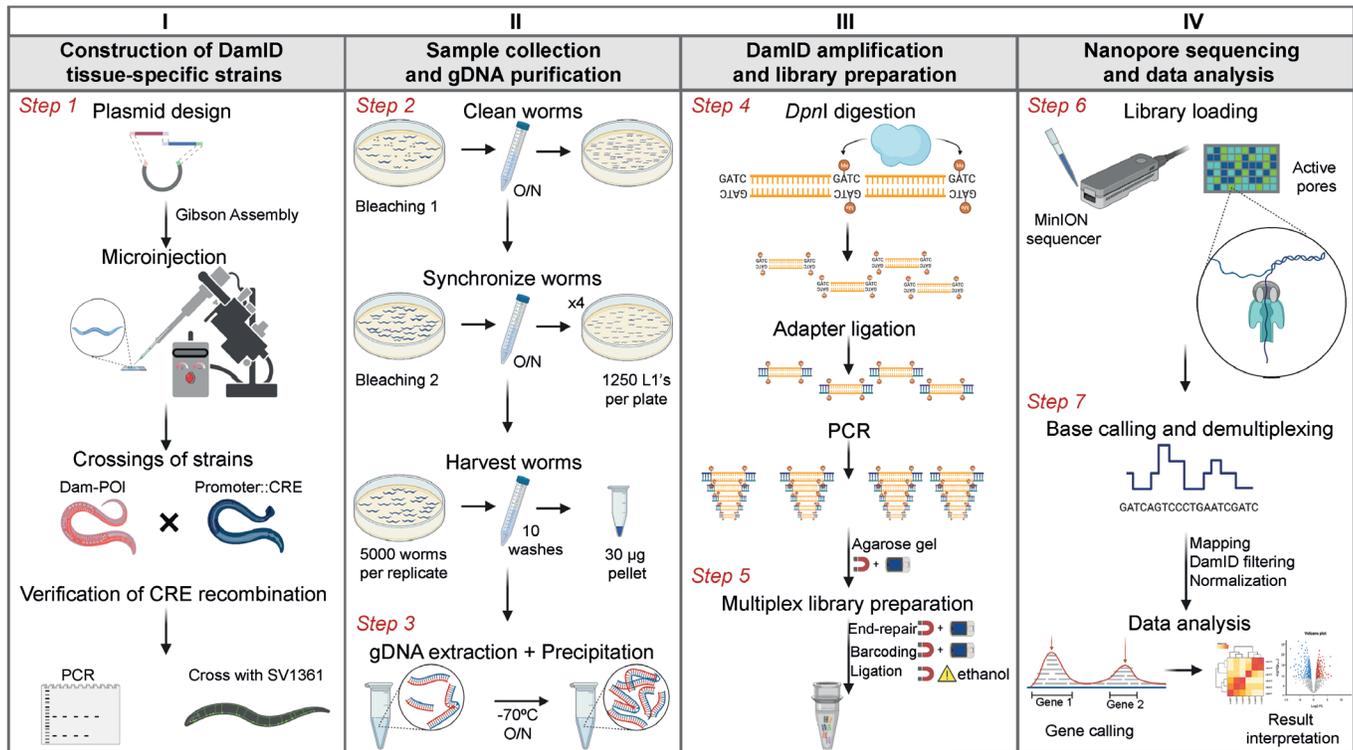
Figure 2. Overview of the entire tissue-specific DamID protocol. The scheme is divided into four main parts: **I.** Construction of DamID tissue-specific plasmids and strains (step 1). **II.** Worm harvesting (step 2) and extraction of gDNA as starting material (step 3). **III.** Amplification of methylated fragments (step 4), purification with magnetic beads (represented with a red magnet), and quantification with Qubit (represented with a blue square) and preparation of multiplex libraries (step 5). **IV.** Sequencing of libraries using Nanopore technology (step 6) and data analysis (step 7).

1. Construction of DamID tissue-specific strains

The tissue-specific promoter driving the expression of CRE protein (promoter::CRE) is selected according to the tissue to be studied (like for the examples in **Fig. 1C**). The CRE expression plasmid can be easily designed with Gibson Assembly technology, allowing the fast and easy analysis of a POI in multiple tissues (**Fig. 3**).

NOTE: Pre-existing CRE expression lines containing single copy transgenes (**Fig. 3** [43,51]) or integrated transgene arrays [52] could potentially be used.

The plasmid expressing Dam-POI should be designed for each protein to analyse, whereas the GFP-Dam vector, pCFJ150 *hsp-16.2p::loxP::mCherry::STOP::loxP::gfp::dam::unc-58 3'UTR* [43], remains constant. Both plasmids contain the *loxP::mCherry::STOP::loxP* cassette.

1.1. Using standard molecular cloning techniques clone the gene of interest into a Dam expression plasmid of choice, *e.g.*, plasmid #503 [pCFJ150 *hsp-16.2p::loxP::mCherry::STOP::loxP::dam::rpb-6::unc-54 3'UTR*] or #418 [pCFJ150 *hsp-16.2p::loxP::mCherry::STOP::loxP::gfp::dam::unc-54 3'UTR*], depending on whether N- or C-terminal tagging is preferred [43,47]. Annotated plasmid sequences are provided as supplementary material (**File S1** and **S2**). The transgene must be under the control of an inducible promoter with low basal activity and a characterized 3′UTR. The plasmids above include the *hsp-16.2* promoter and the *unc-54* 3′UTR (**Fig. 3A**).

1.2. Verify the sequence using primers that cover the insertion sites and the gene of interest. With the plasmids above, primers Dam-forward and *unc-54*-reverse can be used.

1.3. Using standard molecular cloning techniques, such as Gibson Assembly, clone the promoter of interest into a CRE expression plasmid of choice, *e.g.*, pSR33 (**Fig. 3B** [51]).

Figure 3. Design of the plasmids used in tissue-specific DamID. A. Outline of the Dam::POI (protein of interest) plasmid construction using Gibson Assembly technology. Blue arrows represent the Gibson Assembly primers that amplify the gene of interest, violet arrows represent the Gibson Assembly primers that amplify the dam gene. Scissors represent restriction sites for excision of *gfp:dam* fragment in pCFJ150 plasmid. Using Gibson Assembly reaction, the gene of interest (blue) and the dam (violet) fragments are inserted in the pCFJ150 backbone. **B.** Outline of the tissue-specific CRE expression plasmid construction using Gibson Assembly technology. Red arrows represent the Gibson Assembly primers to amplify the tissue-specific promoter of interest. Scissors represent restriction sites for excision of *hsp-16.2p* in pSR33 plasmid. Using a Gibson Assembly reaction, a new promoter fragment (blue) is inserted in the pSR33 backbone.

1.4. Verify the sequence of the insertion. In the pSR33 backbone, M13 forward and M13 reverse primers can be used for sequencing. Depending on the size of the inserted promoter, additional sequencing primers may need to be designed.

1.5. Inject the DamID vectors into the gonad of an appropriate *C. elegans* host strain for MosSCI integration and isolate transgenic lines according to standard MosSCI protocols [50]. Dam-POI constructs are integrated either on chromosome II or IV and promoter::CRE constructs are integrated on chromosome X.

1.6. When designing new CRE drivers to target specific cell types, verify the correct localization and recombination function of CRE *in vivo*, by crossing the promoter::CRE line with the reporter line SV1361 available from CGC [51]. This reporter line ubiquitously expresses a nuclear red marker from the [*rps-27p::loxP::NLS::mCherry::let858 3'UTR::loxP::NLS::GFP::let-858 3'UTR*] transgene. Upon successful recombination of the mCherry cassette, CRE-expressing cells will start to produce NLS::GFP, labeling the corresponding nuclei in green (**Fig. 4B**).

1.7. Cross the strains expressing Dam-POI and promoter::CRE to generate a line being homozygous for both transgenes.

1.8. Using PCRs, verify the Dam-POI recombination causing the *loxP::mCherry::STOP::loxP* cassette excision (**Fig. 4A**). The primers design must ensure that two amplification products are distinguished (**Fig. 1B**): a small one, corresponding to the genomic DNA from the CRE-expressing tissues, for which the mCherry cassette is excised; and a large one, corresponding to the rest of the tissues, in which CRE is absent and the mCherry cassette is still present (**Fig. 4A**).
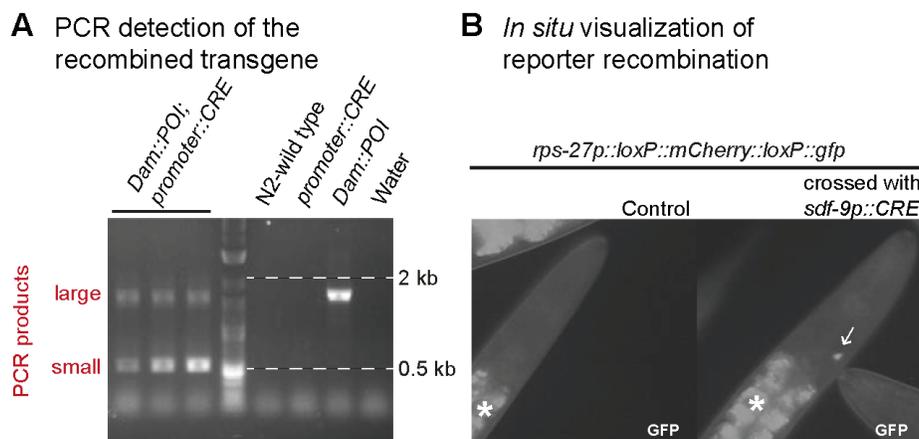
Figure 4. Verification of CRE-mediated recombination. A. Verification of recombination using PCR. Representative agarose gel electrophoresis results, showing three successfully recombined samples (left lanes), a size ladder, and four controls. Primers should be designed to evaluate the presence or the absence of *loxP::mCherry::STOP::loxP* cassette (as schematized in **Fig. 1B**). A successful PCR should generate two amplification products: a small one (about 500 bp), corresponding to the gDNA from the CRE-expressing tissues for which the mCherry cassette is excised; and a large one (about 1700 bp), corresponding to the rest of the tissues in which the mCherry cassette is still present. Note: Although a smaller number of cells are expressing the recombined product, the smaller PCR product tends to be more abundant due to PCR bias favoring the amplification of smaller products. **B.** Verification of CRE-mediated recombination in the selected tissue with a reporter transgene. The depicted example shows the CRE expression in XXX neuroendocrine cells *in vivo*. An XXX-specific CRE driver strain using the *sdf-9p* promoter [DAG827; 43] was crossed with the reporter strain SV1361, which carries a floxed *mCherry::STOP* cassette in front of an *nls::gfp* open reading frame. Upon successful recombination, the *mCherry* cassette is excised and CRE-expressing cells produce NLS::GFP, here labeling XXX neuroendocrine nuclei (white arrow). The asterisks mark autofluorescence of intestinal cells.

2. Nematode culture

2.1. Collect embryos from asynchronous cultures by standard hypochlorite treatment.

2.1.1. Wash 3–4 mixed-stage 6 cm NGM plates containing many gravid adults with M9 and transfer to a 15 ml Falcon tube.

2.1.2. Centrifuge at 400 *g* for 2 min in a swinging bucket centrifuge, and discard the supernatant using a vacuum pump.

2.1.3. Wash animals twice with 14 ml M9 buffer. Each time, centrifuge at 400 *g* for 2 min and discard the supernatant. Repeat if the solution is still cloudy, a sign that bacteria are still present.

2.1.4. Add 2 ml of fresh bleach solution at room temperature (recipe for 5 ml: 3.5 ml Milli-Q water, 1 ml 13% sodium hypochlorite, 0.5 ml 5M KOH). Agitate the tubes by hand and vortex occasionally (no more than 4 min; and no more than 4–6 strains at a time). Check worms regularly with a dissecting stereoscope. When 60% of the worms are broken up, proceed to the next step. In subsequent steps, work under sterile conditions to avoid contaminations with Dam positive bacteria.

2.1.5. Add 13 ml Milli-Q water and completely resuspend the pellet to avoid worm death.

2.1.6. Centrifuge at 800 *g* for 1 min and discard supernatant.

2.1.7. Wash 5 times with 13 ml M9, centrifuge at 800 *g* for 1 min and discard supernatant. After the last wash, resuspend in 5 ml of M9. Count the number of embryos in 2–10 µl aliquots.

2.1.8. Leave embryos to hatch overnight (14–16 h) at 16–20°C with gentle agitation on a roller.

2.1.9. Centrifuge at 600 *g* for 1 min and discard supernatant leaving about 1–1.5 ml to resuspend the L1 larvae.

2.2. Count hatched L1s in 2–10 µl aliquots.

2.3. Place 1500–2000 L1 animals per 10 cm plate containing a thick lawn of Dam negative *E. coli* bacteria (*e.g.*, strain GM48, GM119) as a food source (prepared with 1 ml of a fresh Dam- overnight liquid culture). Set at least two cultures per strain, as biological replicates.

2.4. Incubate worms at 15°C to 20°C and let them grow for two generations, making sure they never run out of food.

2.5. Once the plate contains mainly gravid adults, wash the plates with M9 and transfer the liquid to a 15 ml falcon tube.

2.6. Repeat steps 2.1 and 2.2.

NOTE: The first bleaching is to remove traces of OP50 Dam positive *E. coli*. This strain would create a high background Dam signal as methylated bacterial DNA is co-purified with the worm DNA. Animals are then grown with GM48 Dam negative *E. coli*. We estimate that after 2 generations most remaining bacterial DNA is not methylated. The second bleaching is to synchronize worm growth for the experiment.

2.7. Place 1000–1250 L1s per 10 cm plate containing a thick lawn of Dam negative *E. coli* bacteria (prepared with 1 ml of fresh Dam negative strain grown overnight in LB and left to dry for 5 h to overnight once spread on the plate). Use a total of four 10 cm-plates per strain and per biological replicate, in order to grow a total of 4000–5000 worms for each replicate.

2.8. Incubate worms at 15°C to 20°C and collect animals when they reach the desired life stage (*e.g.*, 74 h for L4; 94 h for non-gravid young adults, *etc.*).

2.9. Harvest worms and wash them 5 times with 15 ml M9 to remove bacteria. Do not centrifuge, but let worms go down by decantation; later wash worms 5–7 more times with M9 and centrifugation at 400 *g* for 1 min. Keep the M9 at 15°C to avoid heat-shocking the animals.

2.10. After the last wash, resuspend the worms in less than 1.5 ml M9 and transfer the suspension to 1.5 ml microcentrifuge tubes. Centrifuge at 13000 *g* for 30 s, discard supernatant and make aliquots containing about 30–35 µl worm material. Remove as much as possible the excess liquid to obtain a worm pellet of about 30 mg, snap-freeze in liquid nitrogen and store at −80°C until needed.

CRITICAL STEPS:

✓ A GFP::Dam or Dam-only control, for each tissue, must be processed in parallel for each replicate.

✓ Low growth temperature (15°C to 20°C) will prevent the induction of *hsp-16.2p*-driven transgenes. We have not evaluated the effect of higher growth temperature, but we consider it might potentially increase methylation signal background noise or trigger Dam::POI toxicity effect in case of higher transgene expression.

✓ Performing a large number of washes and using decantation (instead of centrifugation) in the first five washes is essential to avoid contamination with *E.coli*, which increases background during the DamID PCR (**Fig. 5**).

✓ If many worms are lost during washes as they stick to the plastic walls of the tubes during washing steps, a small amount of detergent (0.01% Tween20) can be added to the M9. The use of glass pipettes similarly increases worm recovery.

PAUSE POINT: Samples can be kept at −80°C until required.

3. Purification of gDNA

3.1. Purify genomic DNA (gDNA) from previously isolated 30 mg worm pellets. Use the DNeasy Blood and Tissue Kit (QIAGEN #69504) with the enclosed protocol for purification of total DNA from Animal Tissues (Spin-Column).

SPECIFIC STEPS:

✓ The DNAeasy column should not be overloaded.

✓ The ATL and AL buffers should be pre-warmed to dissolve precipitates.

✓ All steps should be carried out as gently as possible to avoid gDNA shearing. The sample is only vortexed for the addition of proteinase K.

✓ Lysis with proteinase K is performed for 3 h in a shaking thermomixer, but can be extended for up to 6 h (see critical steps below).

✓ Add 4 µl of RNase A (100 mg/ml) after lysis.

✓ To achieve complete elution of the DNA, the final elution is carried out in two successive rounds, first with 200 µl, then with 100 µl.

3.2. Measure gDNA concentration using Nanodrop or, for more consistent and accurate results, with a DNA fluorometer (Qubit or equivalent).

NOTE: The expected yield is 3–5 µg and the concentration is expected to be below the minimal concentration of 200 ng/µl needed in step 4. Therefore, a concentration step using sodium acetate precipitation (see 3.3) is needed.

3.3. Precipitation with 3M sodium acetate:

3.3.1. Add 0.1 volume of 3M sodium acetate to your sample, and then 3 volumes of 96% ethanol (*e.g.*, for a 300 µl sample, add 30 µl of 3M sodium acetate and 900 µl of 96% ethanol). Incubate at −80°C overnight. Centrifuge 30 min at 4°C at 16000 *g*.

3.3.2. Remove supernatant (as much as possible).

3.3.3. Add 500 fresh and chilled 70% ethanol.

3.3.4. Centrifuge 15 min at 4°C at 16000 *g*.

3.3.5. Remove supernatant (as much as possible).

3.3.6. Let the pellet dry (do not overdry).

3.3.7. Add Milli-Q water or Tris 10 mM pH 8, incubate 1 min at room temperature and resuspend well; aim for a final concentration of about 250 ng/µl.

3.3.8. Measure the concentration with Nanodrop, or better with Qubit or equivalent.

CRITICAL STEPS:

✓ At the L1 or dauer stages, the cuticle is harder and more difficult to lyze. The proteinase K digestion step from the DNeasy Blood and Tissue Kit can be extended to 6 h.

✓ As quality control, 1 µl of gDNA can be run on a 1% agarose gel. gDNA should be visible as a single band at the top of the gel without any smear of lower molecular weight fragments.

PAUSE POINT: gDNA can be stored at −80°C for at least 6 months.
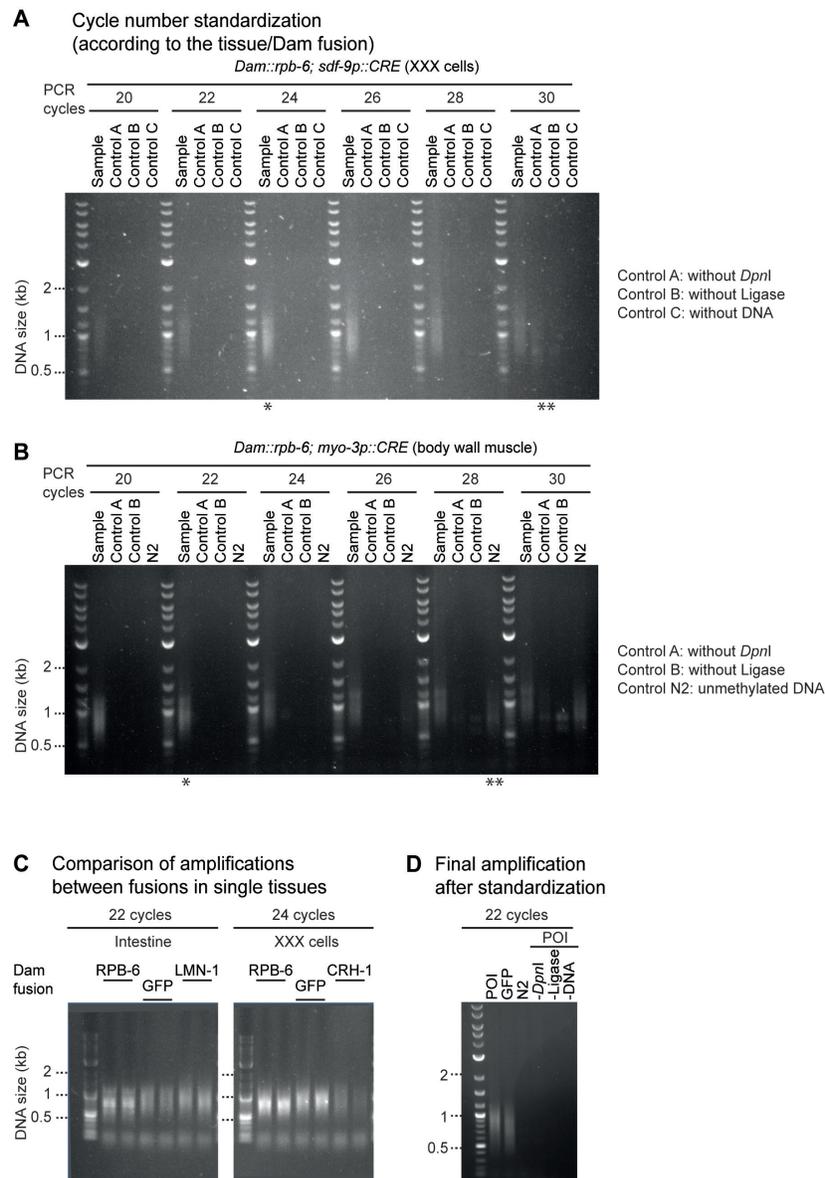
Figure 5. Standardization of DamID PCR. Examples of agarose gel electrophoresis analyses of amplified methylated fragments in tissue-specific DamID samples and required controls (A, sample with methylated DNA, without DpnI but with ligase; B, sample with methylated DNA, with DpnI but without ligase; C, sample with water, DpnI and ligase). The appropriate number of PCR cycles used to amplify the methylated fragments for each Dam-POI has to be determined according to the tissue where it is expressed. **A.** Agarose gel showing the smear signal obtained for Dam::RBP-6 in XXX cells over a range of 20 to 30 cycles. Several controls (A, B and C) are analyzed in parallel to the samples to rule out the production of non-DamID amplicons. The cycle number selection is based on a strong signal for the sample (complete reaction: methylated DNA, DpnI and ligase) and an absence of signal in the controls. **B.** Same as in A for muscle cells. One asterisk indicates the selected number of cycles for each tissue (24 for XXX cells; 22 cycles for muscle cells). The cycle number selection is based on a strong signal for the sample and an absence of signal in the controls (A, B, C, and N2: sample of unmethylated DNA, DpnI and ligase). Two asterisks indicate the cycle where a non-specific signal starts to be visible for one or more of the controls. These might arise from contamination with methylated **E.** coli DNA (control A), gDNA breaks (control B) or contaminated reagents (control C or water), as well as from non-specific amplicons arising from non-methylated worm DNA (control N2). **C.** Agarose gel showing amplicons obtained from DNA of strains expressing different Dam-POI in two different cell types (each time two biological replicates). Left: amplicons obtained from strains with intestine-specific expression of Dam fusions to RPB-6 (RNA polymerase subunit 6), GFP (chromatin accessibility control) and LMN-1 (lamin). The RNA polymerase subunit(RBP-6) produces stronger signals than LMN-1, associated with a shorter proportion of the genome. Right: amplicons obtained from strains with XXX-specific expression of Dam fusions to RPB-6 (RNA polymerase), GFP (chromatin accessibility control) and CRH-1 (a transcription factor). The RNA polymerase subunit (RPB-6) produces a stronger DamID signal than the CRH-1 transcription factor. **D.** Example of successful amplification of Dam-methylated DNA after standardizations. Dam-POI and GFP-Dam experiments were processed and run in parallel with all technical controls. Only a small volume of the preparation was analyzed on gel for this verification and the rest could be used for the DamID experiment. Methylated DNA amplicons are visible as a smear (400–1200 bp range).

4. Digestion and purification of methylated DNA

4.1. Digest 500 ng gDNA using 10 units *Dpn*I (NEB #R0176S; cuts methylated GmATC).

4.1.1. Set up *Dpn*I digestion reactions on ice (**Table 1**). Make sure to prepare additional tubes for controls: (i) without *Dpn*I (one control for each biological sample); (ii) for one of the biological samples with methylated DNA, a second digestion replicate control digested with *Dpn*I, which will be later used without ligase (**Fig. 5A**).

Table 1. *Dpn*I digestion mix.

Component	Volume per reaction (µl)	Final
10× CutSmart	1	
*Dpn*I (20 U/µl)	0.5	
gDNA	X (max. 8.5 µl)	500 ng
Nuclease-free water	q.s. to 10 µl	

4.1.2. Incubate 6 h at 37°C in a thermocycler.

4.1.3. Heat-inactivate the enzyme at 80°C for 20 min and cool at 4°C.

4.2. Prepare double-stranded adapters (ds-adapters, each at 50 µM final concentration).

4.2.1. Mix 50 µl of primer AdRt (100 µM) and 50 µl of AdRb (100 µM).

4.2.2. Anneal the primers in a thermocycler, using the following program: 95°C for 10 min and then reduce 5 degrees every 1 min until 25°C, then 12°C forever (at least 10 min).

NOTE: The ds-adapter mix can be prepared in advance and kept frozen at −20°C.

4.3. Ligate the adapters to *Dpn*I-digested DNA

4.3.1. Prepare the ds-adapter ligation reaction mix on ice (**Table 2**), as well as the control B (see above) with *Dpn*I-digested DNA, but without T4 DNA ligase.

Table 2. Ligation mix.

Component	Volume per reaction (µl)	Final
10× T4 ligase buffer	2	2x
T4 ligase	0.7	
ds-adapters (50 µM)	0.6	3 µM
Nuclease-free water	6.7	

4.3.2. Add 10 µl of the ligation mix to each sample (final volume 20 µl), as well as the 10 µl no ligase control to the control B.

4.3.3. Incubate at 16°C overnight (at least 16 h) in a thermocycler.

4.3.4. Inactivate the T4 DNA ligase by incubating 10 min at 65°C.

4.4. Amplify methylated DNA using HiDi DNA polymerase and Adr primer (**Table 3** and **Table 4**). Include a "no DNA" control in which the DNA template is replaced with water (control C; **Fig. 5A**).

NOTE: Step 4 is subject to optimization in a sample-dependent manner (see critical steps below).

4.5. Verify each PCR reaction by loading 5 µl on a 1% agarose gel. Successful amplification of Dam-methylated DNA should yield a 400–1200 bp smear (**Fig. 5C**).

4.6. Purify the DNA with magnetic AMPure beads, following the AMPure XP 1.8× cleanup protocol detailed below.

4.6.1. For each PCR reaction, transfer 45 µl of PCR product into a 1.5 ml microcentrifuge tube.

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Table 3. PCR-mix.

Component	Volume per reaction (µl)	Final
DNA (from ligation step)	20	
10× HiDi PCR buffer	5	1×
dNTP Mix 2.5 mM	4	0.2 mM
Nuclease-free water	18.75	
Adr (50 µM)	1.25	1.25 µM
HiDi DNA polymerase	1	
Final volume	50	

Table 4. PCR program (about 3 h).

Step	Denaturation	Annealing	Extension
1			68°C for 10 min
2	94°C for 1 min	65°C for 5 min	68°C for 15 min
3 (4×)	94°C for 1 min	65°C for 1 min	68°C for 10 min
4 (20×)	94°C for 1 min	65°C for 1 min	68°C for 2 min

4.6.2. Briefly vortex the AMPure XP beads to resuspend them. Keep them on ice at all times.

4.6.3. Transfer 81 µl of bead suspension (corresponding to 1.8× the sample volume) into the tube containing the sample and pipette up and down 10 times to mix thoroughly. Incubate at room temperature for 5 min.

4.6.4. Transfer the tube to the magnetic stand and let it stand for at least 2 min for the beads to separate. From this step onwards, do not move the tubes from the stand unless explicitly mentioned.

4.6.5. Gently pipette out the cleared solution, always taking care not to disturb the beads.

4.6.6. Add 200 µl of freshly prepared 70% ethanol solution to wash the beads. Incubate for at least 30 s. Remove the supernatant. Repeat twice. (It is advisable to reach the bottom of the tube with the pipette tip and then dispense the solution).

4.6.7. Take the tubes out of the magnetic stand and let it stand for 30–60 s with the lid open so as to let the residual ethanol evaporate.

4.6.8. Elute by adding 30 µl of Milli-Q water or TE into the tube and resuspending the beads by pipetting (10 times).

4.6.9. Incubate at room temperature for 2 min and then place the tubes on the magnetic stand again.

4.6.10. Let the beads separate for 2 min and then slowly pipette out the eluted sample into a fresh microcentrifuge tube, recovering about 26–28 µl of purified DNA. Take care not to collect any bead.

4.7. Quantify DNA using Qubit. Concentration should be at least 20–50 ng/µl and the total amount > 0.08 pmol.

CRITICAL STEPS:

✓ Do not vortex the sample at any step and only mix gently with a pipet to avoid shearing the gDNA.

✓ All primers are HPLC-purified (IDT, Coralville, USA).

✓ The first step of the PCR is an elongation from the ligated adapters. Therefore, the first step of the PCR is a 10 min incubation at 68°C, not a denaturation at 94°C.

✓ It is useful to prepare double-stranded adapters in advance and keep them stored at −20°C (stored maximum 1 year).

✔ We currently use T4 ligase HC from Thermo Fisher (EL0013).

✔ Step 4 is performed twice, first to standardize the number of PCR-cycles, which is optimized for each POI::Dam expressed in a different tissue (**Fig. 5A** and **5B**); and second to perform the experiment and continue with step 5 (**Fig. 5C** and **5D**).

NOTE: To standardize the number of PCR-cycles, only change the last step in the PCR program. Collect 8 μl of the amplicon after every 2 cycles ranging from cycle number 18 to 28 (last cycle); at cycle 28 background signal in the N2 control begins to appear (**Fig. 5B**).

PAUSE POINT: Purified methylated fragments can be stored at −20°C up to 6 months.

5. Multiplexed library preparation for DamID Nanopore sequencing

The library preparation of DamIDseq uses the native barcoding genomic DNA workflow. In short, amplicon ends are first repaired to create blunt-ended fragments and a single dA is added on both strands at the 3' ends (step 5.1). This dA overhang is then ligated to a dT-tailed barcoded adapter (step 5.2). After purification, barcoded amplicons are pooled together in equimolar amounts and ligated to the sequencing adapter bound to the motor protein necessary for nanopore sequencing (step 5.3).

5.1. End-repair

5.1.1. For each methylated fragment PCR sample, add 0.12 pmol diluted in 25 μl of Milli-Q water in a 0.2 ml PCR tube. Prepare the End-repair master mix (multiply by [n samples + 0.2] to compensate for pipetting errors during the distribution in single tubes) on ice and add 5 μl of the mix to each amplicon sample (**Table 5**).

NOTE: To calculate the pmols of each sample, we consider an average length of 2000 bp for the amplicon size and calculate the molarity of the purified DNA using the online tool dsDNA: Mass to/from Moles Convertor (https://nebiocalculator.neb.com/).

Table 5. End-repair master mix.

Component	Volume per reaction (μl)
0.12 pmol amplicon	25
Ultra II End-prep buffer	3.5
Ultra II End-prep enzyme	1.5
Final volume	30

5.1.2. Add 5 μl of the End-repair master mix to each 25 μl diluted amplicon PCR tube.

5.1.3. Mix gently by flicking the PCR tube, and quick spin to recover droplets.

5.1.4. Incubate at 20°C for 5 min in a thermocycler.

5.1.5. Inactivate the enzyme by incubating at 65°C for 5 min in a thermocycler.

5.1.6. Purify using magnetic beads:

5.1.7. (i) Add 1.8× volume (54 μl) of well resuspended AMPure XP beads. (ii) Incubate 10 min on the rotator mixer for binding. (iii) Spin down the sample and pellet on a magnetic rack. Incubate at least 5 min on the magnetic rack for the beads to separate. From this step onwards, do not move the tubes from the stand unless explicitly mentioned. (iv) Gently pipette out the cleared solution, always taking care not to disturb the beads. (v) Add 200 μl of freshly prepared 80% ethanol solution to wash the beads. Incubate for at least 30 s and pipette out the cleared solution. (It is advisable to reach the bottom of the tube with the pipette tip and then dispense the solution). (vi) Repeat twice making sure no ethanol droplet is left on the tube walls. (vii) Take the tubes out of the magnetic stand and let them dry for 5 min with the lid open so as to let the residual ethanol evaporate. (viii) Elute with 11 μl of Milli-Q water into each tube and resuspend the

beads by pipetting (10 times). (ix) Incubate at room temperature for 2 min and then place the tubes on the magnetic stand again. (x) Let the beads separate for 2 min and then slowly pipette out the eluted sample into a new 0.2 ml PCR tube, recovering about 10.5 µl of purified DNA. Take care not to collect any bead with the eluate.

5.1.8. Use 1 µl to quantify DNA with Qubit. At least 50%–60% of input should be recovered.

CRITICAL STEPS:

✓ Always set up the reactions on ice and perform the purification with magnetic beads at room temperature.

✓ Be careful not to over-dry the magnetic pellet, because this may impede its complete resuspension.

✓ It is advisable to finish the libraries on the same day, calculate the time well, considering the multiple purification steps and quantifications with Qubit.

✓ It is advisable to write down the concentrations obtained in each step, as well as essential annotations, for example, the barcode used for each amplicon (next step).

5.2. Barcoding

5.2.1. Carefully record the barcode attribution for each amplicon sample.

5.2.2. Thaw barcodes at room temperature.

5.2.3. For each amplicon, prepare the mix on ice using a 0.2 ml PCR tube (**Table 6**).

5.2.4. Mix gently by flicking the PCR tube, and quick spin to recover droplets.

5.2.5. Incubate 10 min at 25°C using a thermocycler.

5.2.6. Purify using well resuspended magnetic beads (1.8× volume = 36 µl beads). Incubate 10 min on the rotator mixer for binding and incubate at least 5 min on the magnet rack for bead collection.

5.2.7. Spin down the sample and pellet on the magnet rack. Incubate at least 5 min on the magnet rack for bead collection.

5.2.8. Gently pipette out the cleared solution, always taking care not to disturb the beads.

5.2.9. Add 200 µl of freshly prepared 80% ethanol solution to wash the beads. Incubate for at least 30 s and pipette out the cleared solution. (It is advisable to reach the bottom of the tube with the pipette tip and then dispense the solution).

5.2.10. Repeat twice making sure no ethanol droplet is left on the tube walls.

5.2.11. Take the tubes out of the magnetic stand and let them dry for 5 min with the lid open so as to let the residual ethanol evaporate.

5.2.12. Elute with 11.5 µl of Milli-Q water into each tube and resuspend the beads by pipetting (10 times).

5.2.13. Incubate at room temperature for 2 min and then place the tubes on the magnetic stand again.

5.2.14. Let the beads separate for 2 min and then slowly pipette out the eluted sample into a new 0.2 ml PCR tube, recovering about 11 µl of purified DNA. Take care not to collect any beads with the eluate.

5.2.15. Use 1 µl to quantify DNA with Qubit. You should recover > 0.011 pmol.

Table 6. Barcoding mix.

Component	Amount per reaction (µl)
End-repaired amplicons	9
Barcode (ONT)	1
NEB/Blunt TA master mix	10
Final volume	20

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CRITICAL STEPS:

✓ For the barcoding mix, use as much as possible End-repaired amplicons, with a maximum of 0.038 pmol.

✓ Always perform the reactions on ice and the purification with magnetic beads at room temperature.

✓ Be careful not to over-dry the magnetic pellet, because this may impede its complete resuspension.

TROUBLESHOOTING: If the end-repaired amplicon concentration is too low, the barcoding reaction can be scaled up 1.5 times (using 13.5 µl of end-repaired amplicons).

5.3. Multiplexed library preparation

5.3.1. Using a DNA low-binding 1.5 ml tube, combine equivalent amounts (same mass) of the different libraries in 65 µl final volume, adding water to complete.

NOTE: To calculate the pmols of each sample, we consider an average length of 1000 bp for the amplicon size and calculate the molarity of the purified DNA using the online tool dsDNA: Mass to/from Moles Convertor (https://nebiocalculator.neb.com/). At this stage, there is only one tube for several libraries. You can typically combine up to 12 libraries, ensuring that sufficient samples are combined to produce a pooled sample comprising at least 0.15 pmol of each library, but without exceeding a total mass of 1.5 µg DNA.

5.3.2. Prepare the adapter ligation mix on ice (**Table 7**).

5.3.3. Incubate 10 min at 26°C in a thermocycler.

5.3.4. Purify the library with magnetic AMPure XP beads: (i) Add 1.2× volume (120 µl) of well-re-suspended magnetic beads. (ii) Incubate 5 min on the rotator mixer for binding. (iii) Spin down the sample and pellet on the magnet rack. Incubate at least 5 min on the magnet rack for bead collection and remove the supernatant. (iv) Wash beads with 250 µl of SFB buffer (ONT), not ethanol (critical step). Resuspend by flicking well. (v) Spin down the sample and pellet on the magnet rack for at least 5 min. (vi) Repeat wash with SFB buffer. (vii) Spin down the sample and pellet on the magnet rack for at least 5 min. (viii) Remove residual supernatant. (ix) Dry 30 s after the second wash, remove droplets on the sides of the tubes using vacuum suction. Do not overdry the pellet. (x) Remove from magnetic rack, elute and resuspend in 15 µl EB (ONT). (xi) Incubate 10 min at room temperature. (xii)Put in the magnetic rack for pellet formation, transfer 14.5 µl in a DNA low-binding PCR tube and keep on ice. The library is ready to load onto the flow cell.

Table 7. Adapter ligation mix.

Component	Volume per reaction (µl)
Pooled barcode sample	65
Adapter Mix II (AMII, ONT)	5
NEBNext ligase buffer (5×)	20
Quick T4 DNA Ligase	10
Final volume	100

CRITICAL STEPS:

✓ SFB and EB buffers should always be kept on ice once thawed.

✓ Washes should be performed with an SFB buffer. Ethanol can denature the motor protein on the sequencing adapters, producing pore occupancy close to 0. Therefore it is necessary to make the wash step after sequencing adapter ligation with the SFB buffer. SFB (Short Fragment Buffer) is used to retain DNA fragments of all sizes. If you need to enrich DNA fragments of 3 kb or longer, it is recommended to use LFB (Long Fragment Buffer). This purification is the most critical step in the protocol.

✓ Optionally the library can be quantified to check the DNA concentration. The total amount should be about 430 ng.

TROUBLESHOOTING: For high molecular weight DNA, incubating at 37ºC with EB can improve the recovery of long fragments.

6. Sequencing

6.1. Priming and loading the spotON flow cell. A detailed protocol can be download from the ONT website (https://community.nanoporetech.com/):

6.1.1. Thaw the Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT) and one tube of Flush Buffer (FB) at room temperature and thereafter keep the tubes on ice.

6.1.2. Mix the SQB and FB tubes by vortexing, and FLT by pipetting, spin down and return to ice.

6.1.3. Open the lid of the nanopore sequencing device and slide the flow cell's priming port cover clockwise so that the priming port is visible.

6.1.4. Check for any small bubble under the cover.

If needed, draw back a small volume to remove any bubble (a few μls) as follows:

✓ Set a P1000 pipette to 200 μl.

✓ Insert the tip into the priming port.

✓ Turn the wheel until the dial shows 220–230 μl, or until a small volume of buffer enters the pipette tip.

✓ Visually check that there is a continuous buffer from the priming port across the sensor array.

✓ Check for any small bubble under the cover.

6.1.5. Prepare the flow cell priming mix: Add 30 μl of FLT directly to the tube of FB, and mix by pipetting up and down.

6.1.6. Load 800 μl of the priming mix into the flow cell *via* the priming port, avoiding the introduction of air bubbles (with spotON closed). Wait for 5 min.

6.1.7. Thoroughly mix the contents of LB by pipetting.

6.1.8. In a new tube, prepare the library mix for loading (**Table 8**).

6.1.9. Complete the flow cell priming. Open the spotON port.

6.1.10. Add 200 μl of priming mix to the priming port (not the spotON port). Avoid the introduction of air bubbles.

6.1.11. Mix the library by pipetting and add 75 μl in dropwise fashion to the spotON port.

6.1.12. Close spotON and priming ports.

Table 8. Library sequencing mix.

Component	Volume per sample (μl)
SQB (ONT)	37.5
LB (ONT)	25.5
Pooled library	12
Final volume	75

6.2. Starting sequencing run.

6.2.1. Turn on MinIT/computer/plug Minion.

6.2.2. Choose the flow cell type and check the "Available" box.

6.2.3. Make a hardware check of the Minion using the Configuration Test Cell. The flow cell must have >1000 active pores.

6.2.4. Click the "New Experiment" button at the bottom left of the GUI.

6.2.5. Select the running parameters for your experiment (FASTQ or FAST5).

6.2.6. Click "Start run". We estimate that a good library should have at least 10 million reads after an overnight run.

CRITICAL STEPS:

✓ The array of pores must be covered by buffer at all times. When you remove more than 20–30 µl (step 6.1.4) you can damage the pores in the array.

✓ Perform all pipetting with great care not to introduce bubbles.

✓ The LB tube contains a suspension of beads. It is vital that they are mixed immediately before use.

✓ If you want to reuse the flow cell, please follow the manufacturer's Wash Kit instructions after the sequencing experiment, and store the washed flow cell at 2°C to 8°C.

7. Sequencing data analysis

Oxford Nanopore raw signal data are stored in a compressed format called fast5. Before using it, the base sequence has to be extracted from this raw signal in a process called basecalling. Additionally, as one flow cell is sufficient to sequence at least 8 different libraries at once using barcoding, each sequence has to be assigned to a given barcode, a process called demultiplexing. The following sequence analysis steps contain the parameters used for the analysis in [43] and are available at https://github.com/CellFateNucOrg/basic_ont/releases/tag/v1.0, including the ad hoc R filtering script.

7.1. Basecalling

7.1.1. Basecalling is carried out using a continuously developed software provided by ONT called Guppy. Guppy works best on computers with Graphics Processing Units (GPUs) and can be downloaded for free from the Community webpage at Oxford Nanopore (https://community.nanoporetech.com/downloads [requires free registration]). ONT provides up-to-date models (called configurations) for the raw signal to base conversion, depending on the flow cell version. The basecaller also provides options for quality score filtering of the basecalled sequences. A minimal quality score of 3 is a good compromise between sequence quality and the number of recovered sequences.

7.1.2. `guppy_basecaller`:

`--input_path` insert input_path;
`--save_path insert` destination fastq path;
`-c` insert location of the configuration file;
`--records_per_fastq` insert the number of reads per output fastq file;
`--compress_fastq` #destination fastq file should be gzip compressed;
`--recursive` #guppy will look for fast5 files in all folders inside the input path;
`--qscore_filtering` #guppy will apply basecall quality score filtering;
`--min_qscore 3` #a quality score of 3 is required for the sequence to be kept;
`--device auto` #if using GPU, guppy will chose automatically available GPUs.

7.2. Quality check

7.2.1. Global read qualities can be assessed using pycoQC, a QC mapper specifically developed for ONT sequencing data. pycoQC is freely available here: https://pypi.org/project/pycoQC/.

7.2.2. `pycoQC`:

`-f` insert fastq directory/sequencing_summary.txt;
`-o` insert path and name of QC file;
`--report_title` name the report;
`--min_pass_qual 3` #this is the min_qscore applied above.

7.3. Demultiplexing

7.3.1. Splitting the sequences based on their barcode is done using another part of guppy, the guppy-barcoder command. guppy-barcoder requires the user to enter the name of the barcoding kit and will output the number of sequences for each output and a folder for each barcode which was found.

7.3.2. `guppy_barcoder`:

`-i` insert fastq directory;
`-s` insert barcoded destination fastq directory;
`--recursive` #guppy looks for fastq files in folders inside the fastq directory;
`-q 40000` #number of reads per barcoded fastq file;
`--compress_fastq` #destination fastq file should be gzip compressed; (vi) --device auto #if using GPU, guppy will chose automatically available GPUs;
`--barcode_kits` "EXP-NBD104" #barcoding kit used.

7.4. Read mapping

7.4.1. Although a number of read mappers have been initially used for long read sequences, the current best in class is minimap2 [53] and can be obtained here: https://github.com/lh3/minimap2. As for basecalling, a threshold for mapping quality is required, classically put to 30. Minimap2 requires a genome file to map to in fasta format.

7.4.2. `minimap2`:

`-ax map-ont` #minimap can map short or different types of long reads. This is the option for ONT reads;
`genomeFile` #fasta file with chromosomal sequences;
`path_to_fastq_files` #provide the path and file names for fastq files;
The result can be directly piped to samtools for quality filtering
`| samtools view -q 30 -u -` #the -u option outputs uncompressed bam files for fast subsequent use and pipe for sorting `| samtools sort -T` insert temporary file name `pass_${bc} -o` insert output folder/name.

7.5. DamID Read filtering

7.5.1. DamID amplicons should start and end with a GATC motif. If one or both ends does not, the amplicon likely originates from the ligation of a DNA break to the DamID adapter (step 1.27 above). Sequencing reads are therefore filtered based on the presence of a GATC close to the end of the read, as the sequences sometimes align with neighbouring bases and do not exactly end at the GATC. We consider reads for which both ends fall +/- 8 bp to a GATC as valid DamID reads. All other reads are discarded. Filtering is done using a custom R script.

7.6. DamID signal determination

7.6.1. The DamID profile is classically calculated as the log2 ratio between the Dam signal of the protein of interest and a freely moving Dam, here fused to GFP (this method is described as rpm normalization). This normalizes for chromatin accessibility, as DNA in euchromatic regions is more accessible and will therefore be easier to methylate than DNA in less accessible heterochromatic regions. The pipeline described in [54] can be used for C. elegans, using the bam files generated by the previous step as starting point. It can be downloaded from https://github.com/owenjm/damidseq_pipeline. As mentioned in [43], this is done with parameters: `-bamfiles -extend_reads = 0 --norm_method rpm`.

7.6.2. For certain Dam fusion proteins, the DamID profile is highly correlated with open chromatin (*e.g.*, RNA polymerase since transcribed genes are typically open chromatin accessible to the free Dam). If the classical normalization method were to be used, this would lead to an artificial reduction of the Dam signal [discussed in detail in 54]. To mitigate this problem, the above-mentioned pipeline first provides a value for the correlation between the free Dam and the POI Dam fusion libraries. If libraries are highly correlated ($R > 0.7$), an alternative method based on kernel normalization is used to calculate the log2 ratio between the Dam fusion and the free Dam signal [54] (option `--norm_method kde`).

7.6.3. For all normalization methods, the damid-seq pipeline directly provides a normalized profile in bedGraph format, which can be visualized in a genome browser such as igv (http://software.broadinstitute.org/software/igv/) or used for downstream analysis.

7.7. Gene level DamID values

When analyzing DamID profiles of RNA polymerase, one would want to get an average gene-level DamID value. An easy solution is provided as part of the damid-seq pipeline here: https://github.com/owenjm/polii.gene.call. Using the bedGraph output of the damid-seq pipeline and a General Feature Format (GFF) list of genes (a simple file with chromosome, start, end, strand and name for each gene), gene-levels DamID values are determined.

References

1. Kaletsky R, Yao V, Williams A, Runnels AM, Tadych A, Zhou S, et al. Transcriptome analysis of adult Caenorhabditis elegans cells reveals tissue-specific gene and isoform expression. PLoS Genet. 2018 Aug;14(8):e1007559. https://doi.org/10.1371/journal.pgen.1007559 PMID:30096138

2. Haenni S, Ji Z, Hoque M, Rust N, Sharpe H, Eberhard R, et al. Analysis of C. elegans intestinal gene expression and polyadenylation by fluorescence-activated nuclei sorting and 3′-end-seq. Nucleic Acids Res. 2012 Jul;40(13):6304–18. https://doi.org/10.1093/nar/gks282 PMID:22467213

3. Ma X, Zhan G, Sleumer MC, Chen S, Liu W, Zhang MQ, et al. Analysis of C. elegans muscle transcriptome using trans-splicing-based RNA tagging (SRT). Nucleic Acids Res. 2016 Dec;44(21):e156. PMID:27557708

4. Blazie SM, Babb C, Wilky H, Rawls A, Park JG, Mangone M. Comparative RNA-Seq analysis reveals pervasive tissue-specific alternative polyadenylation in Caenorhabditis elegans intestine and muscles. BMC Biol. 2015 Jan;13(1):4. https://doi.org/10.1186/s12915-015-0116-6 PMID:25601023

5. van Steensel B, Henikoff S. Identification of in vivo DNA targets of chromatin proteins using tethered dam methyltransferase. Nat Biotechnol. 2000 Apr;18(4):424–8. https://doi.org/10.1038/74487 PMID:10748524

6. Woolcock KJ, Gaidatzis D, Punga T, Bühler M. Dicer associates with chromatin to repress genome activity in Schizosaccharomyces pombe. Nat Struct Mol Biol. 2011 Jan;18(1):94–9. https://doi.org/10.1038/nsmb.1935 PMID:21151114

7. Steglich B, Filion GJ, van Steensel B, Ekwall K. The inner nuclear membrane proteins Man1 and Ima1 link to two different types of chromatin at the nuclear periphery in S. pombe. Nucleus. 2012 Jan-Feb;3(1):77–87. https://doi.org/10.4161/nucl.18825 PMID:22156748

8. McCann TS, Guo Y, McDonald WH, Tansey WP. Antagonistic roles for the ubiquitin ligase Asr1 and the ubiquitin-specific protease Ubp3 in subtelomeric gene silencing. Proc Natl Acad Sci USA. 2016 Feb;113(5):1309–14. https://doi.org/10.1073/pnas.1518375113 PMID:26787877

9. Flury V, Georgescu PR, Iesmantavicius V, Shimada Y, Kuzdere T, Braun S, et al. The Histone Acetyltransferase Mst2 Protects Active Chromatin from Epigenetic Silencing by Acetylating the Ubiquitin Ligase Brl1. Mol Cell. 2017 Jul;67(2):294–307.e9. https://doi.org/10.1016/j.molcel.2017.05.026 PMID:28648780

10. Spasskaya DS, Nadolinskaia NI, Tutyaeva VV, Lysov YP, Karpov VL, Karpov DS. Yeast Rpn4 Links the Proteasome and DNA Repair via RAD52 Regulation. Int J Mol Sci. 2020 Oct;21(21):E8097. https://doi.org/10.3390/ijms21218097 PMID:33143019

11. Towbin BD, González-Aguilera C, Sack R, Gaidatzis D, Kalck V, Meister P, et al. Step-wise methylation of histone H3K9 positions heterochromatin at the nuclear periphery. Cell. 2012 Aug;150(5):934–47. https://doi.org/10.1016/j.cell.2012.06.051 PMID:22939621

12. Sharma R, Jost D, Kind J, Gómez-Saldivar G, van Steensel B, Askjaer P, et al. Differential spatial and structural organization of the X chromosome underlies dosage compensation in C. elegans. Genes Dev. 2014 Dec;28(23):2591–6. https://doi.org/10.1101/gad.248864.114 PMID:25452271

13. Schuster E, McElwee JJ, Tullet JM, Doonan R, Matthijssens F, Reece-Hoyes JS, et al. DamID in C. elegans reveals longevity-associated targets of DAF-16/FoxO. Mol Syst Biol. 2010 Aug;6(1):399. https://doi.org/10.1038/msb.2010.54 PMID:20706209

14. Van de Walle P, Muñoz-Jiménez C, Askjaer P, Schoofs L, Temmerman L. DamID identifies targets of CEH-60/PBX that are associated with neuron development and muscle structure in Caenorhabditis elegans. PLoS One. 2020 Dec;15(12):e0242939. https://doi.org/10.1371/journal.pone.0242939 PMID:33306687

15. Muñoz-Jiménez C, Ayuso C, Dobrzynska A, Torres-Mendéz A, Ruiz PC, Askjaer P. An Efficient FLP-Based Toolkit for Spatiotemporal Control of Gene Expression in Caenorhabditis elegans. Genetics. 2017 Aug;206(4):1763–78. https://doi.org/10.1534/genetics.117.201012 PMID:28646043

16. Cabianca DS, Muñoz-Jiménez C, Kalck V, Gaidatzis D, Padeken J, Seeber A, et al. Active chromatin marks drive spatial sequestration of heterochromatin in C. elegans nuclei. Nature. 2019 May;569(7758):734–9. https://doi.org/10.1038/s41586-019-1243-y PMID:31118512

17. Harr JC, Schmid CD, Muñoz-Jiménez C, Romero-Bueno R, Kalck V, Gonzalez-Sandoval A, et al. Loss of an H3K9me anchor rescues laminopathy-linked changes in nuclear organization and muscle function in an Emery-Dreifuss muscular dystrophy model. Genes Dev. 2020 Apr;34(7-8):560–79. https://doi.org/10.1101/gad.332213.119 PMID:32139421

18. Pickersgill H, Kalverda B, de Wit E, Talhout W, Fornerod M, van Steensel B. Characterization of the Drosophila melanogaster genome at the nuclear lamina. Nat Genet. 2006 Sep;38(9):1005–14. https://doi.org/10.1038/ng1852 PMID:16878134

19. Orian A, van Steensel B, Delrow J, Bussemaker HJ, Li L, Sawado T, et al. Genomic binding by the Drosophila Myc, Max, Mad/Mnt transcription factor network. Genes Dev. 2003 May;17(9):1101–14. https://doi.org/10.1101/gad.1066903 PMID:12695332

20. Ilyin AA, Ryazansky SS, Doronin SA, Olenkina OM, Mikhaleva EA, Yakushev EY, et al. Piwi interacts with chromatin at nuclear pores and promiscuously binds nuclear transcripts in Drosophila ovarian somatic cells. Nucleic Acids Res. 2017 Jul;45(13):7666–80. https://doi.org/10.1093/nar/gkx355 PMID:28472469

21. Flint Brodsly N, Bitman-Lotan E, Boico O, Shafat A, Monastirioti M, Gessler M, et al. The transcription factor Hey and nuclear lamins specify and maintain cell identity. eLife. 2019 Jul;8:e44745. https://doi.org/10.7554/eLife.44745 PMID:31310235

22. Estacio-Gómez A, Hassan A, Walmsley E, Le LW, Southall TD. Dynamic neurotransmitter specific transcription factor expression profiles during Drosophila development. Biol Open. 2020 Jun;9(5):bio052928. https://doi.org/10.1242/bio.052928 PMID:32493733

23. Hassan A, Araguas Rodriguez P, Heidmann SK, Walmsley EL, Aughey GN, Southall TD. Condensin I subunit Cap-G is essential for proper gene expression during the maturation of post-mitotic neurons. eLife. 2020 Apr;9:e55159. https://doi.org/10.7554/eLife.55159 PMID:32255428

24. Gutierrez-Triana JA, Mateo JL, Ibberson D, Ryu S, Wittbrodt J. iDamIDseq and iDEAR: an improved method and computational pipeline to profile chromatin-binding proteins. Development. 2016 Nov;143(22):4272–8. https://doi.org/10.1242/dev.139261 PMID:27707796

25. Germann S, Juul-Jensen T, Letarnec B, Gaudin V. DamID, a new tool for studying plant chromatin profiling in vivo, and its use to identify putative LHP1 target loci. Plant J. 2006 Oct;48(1):153–63. https://doi.org/10.1111/j.1365-313X.2006.02859.x PMID:16972870

26. Bollier N, Sicard A, Leblond J, Latrasse D, Gonzalez N, Gévaudant F, et al. At-MINI ZINC FINGER2 and Sl-INHIBITOR OF MERISTEM ACTIVITY,

a Conserved Missing Link in the Regulation of Floral Meristem Termination in Arabidopsis and Tomato. Plant Cell. 2018 Jan;30(1):83–100. https://doi.org/10.1105/tpc.17.00653 PMID:29298836

27. Alvarez JM, Schinke AL, Brooks MD, Pasquino A, Leonelli L, Varala K, et al. Transient genome-wide interactions of the master transcription factor NLP7 initiate a rapid nitrogen-response cascade. Nat Commun. 2020 Mar;11(1):1157. https://doi.org/10.1038/s41467-020-14979-6 PMID:32123177

28. Tosti L, Ashmore J, Tan BS, Carbone B, Mistri TK, Wilson V, et al. Mapping transcription factor occupancy using minimal numbers of cells in vitro and in vivo. Genome Res. 2018 Apr;28(4):592–605. https://doi.org/10.1101/gr.227124.117 PMID:29572359

29. Szczesnik T, Ho JW, Sherwood R. Dam mutants provide improved sensitivity and spatial resolution for profiling transcription factor binding. Epigenetics Chromatin. 2019 Jun;12(1):36. https://doi.org/10.1186/s13072-019-0273-x PMID:31196130

30. Gatticchi L, de Las Heras JI, Roberti R, Schirmer EC. Optimization of DamID for use in primary cultures of mouse hepatocytes. Methods. 2019 Mar;157:88–99. https://doi.org/10.1016/j.ymeth.2018.11.005 PMID:30445179

31. Yattah C, Hernandez M, Huang D, Park H, Liao W, Casaccia P. Dynamic Lamin B1-Gene Association During Oligodendrocyte Progenitor Differentiation. Neurochem Res. 2020 Mar;45(3):606–19. https://doi.org/10.1007/s11064-019-02941-y PMID:32020491

32. Guelen L, Pagie L, Brasset E, Meuleman W, Faza MB, Talhout W, et al. Domain organization of human chromosomes revealed by mapping of nuclear lamina interactions. Nature. 2008 Jun;453(7197):948–51. https://doi.org/10.1038/nature06947 PMID:18463634

33. Vogel MJ, Guelen L, de Wit E, Peric-Hupkes D, Lodén M, Talhout W, et al. Human heterochromatin proteins form large domains containing KRAB-ZNF genes. Genome Res. 2006 Dec;16(12):1493–504. https://doi.org/10.1101/gr.5391806 PMID:17038565

34. Robson MI, de Las Heras JI, Czapiewski R, Sivakumar A, Kerr AR, Schirmer EC. Constrained release of lamina-associated enhancers and genes from the nuclear envelope during T-cell activation facilitates their association in chromosome compartments. Genome Res. 2017 Jul;27(7):1126–38. https://doi.org/10.1101/gr.212308.116 PMID:28424353

35. de Las Heras JI, Zuleger N, Batrakou DG, Czapiewski R, Kerr AR, Schirmer EC. Tissue-specific NETs alter genome organization and regulation even in a heterologous system. Nucleus. 2017 Jan;8(1):81–97. https://doi.org/10.1080/19491034.2016.1261230 PMID:28045568

36. Aughey GN, Estacio Gomez A, Thomson J, Yin H, Southall TD. CATaDa reveals global remodelling of chromatin accessibility during stem cell differentiation in vivo. eLife. 2018 Feb;7:e32341. https://doi.org/10.7554/eLife.32341 PMID:29481322

37. Kind J, Pagie L, Ortabozkoyun H, Boyle S, de Vries SS, Janssen H, et al. Single-cell dynamics of genome-nuclear lamina interactions. Cell. 2013 Mar;153(1):178–92. https://doi.org/10.1016/j.cell.2013.02.028 PMID:23523135

38. Cheetham SW, Gruhn WH, van den Ameele J, Krautz R, Southall TD, Kobayashi T, et al. Targeted DamID reveals differential binding of mammalian pluripotency factors. Development. 2018 Oct;145(20):dev170209. https://doi.org/10.1242/dev.170209 PMID:30185410

39. Hass MR, Liow HH, Chen X, Sharma A, Inoue YU, Inoue T, et al. SpDamID: Marking DNA Bound by Protein Complexes Identifies Notch-Dimer Responsive Enhancers. Mol Cell. 2016 Oct;64(1):213. https://doi.org/10.1016/j.molcel.2016.09.035 PMID:27716485

40. Cheetham SW, Brand AH. RNA-DamID reveals cell-type-specific binding of roX RNAs at chromatin-entry sites. Nat Struct Mol Biol. 2018 Jan;25(1):109–14. https://doi.org/10.1038/s41594-017-0006-4 PMID:29323275

41. Redolfi J, Zhan Y, Valdes-Quezada C, Kryzhanovska M, Guerreiro I, Iesmantavicius V, et al. DamC reveals principles of chromatin folding in vivo without crosslinking and ligation. Nat Struct Mol Biol. 2019 Jun;26(6):471–80. https://doi.org/10.1038/s41594-019-0231-0 PMID:31133702

42. Southall TD, Gold KS, Egger B, Davidson CM, Caygill EE, Marshall OJ, et al. Cell-type-specific profiling of gene expression and chromatin binding without cell isolation: assaying RNA Pol II occupancy in neural stem cells. Dev Cell. 2013 Jul;26(1):101–12. https://doi.org/10.1016/j.devcel.2013.05.020 PMID:23792147

43. Gómez-Saldivar G, Osuna-Luque J, Semple JI, Glauser DA, Jarriault S, Meister P. Tissue-Specific Transcription Footprinting Using RNA Pol DamID (RAPID) in Caenorhabditis elegans. Genetics. 2020 Dec;216(4):931–45. https://doi.org/10.1534/genetics.120.303774 PMID:33037050

44. Rooijers K, Markodimitraki CM, Rang FJ, de Vries SS, Chialastri A, de Luca KL, et al. Simultaneous quantification of protein-DNA contacts and transcriptomes in single cells. Nat Biotechnol. 2019 Jul;37(7):766–72. https://doi.org/10.1038/s41587-019-0150-y PMID:31209373

45. Markodimitraki CM, Rang FJ, Rooijers K, de Vries SS, Chialastri A, de Luca KL, et al. Simultaneous quantification of protein-DNA interactions and transcriptomes in single cells with scDam&T-seq. Nat Protoc. 2020 Jun;15(6):1922–53. https://doi.org/10.1038/s41596-020-0314-8 PMID:32350457

46. van Dijk EL, Jaszczyszyn Y, Naquin D, Thermes C. The Third Revolution in Sequencing Technology. Trends Genet. 2018 Sep;34(9):666–81. https://doi.org/10.1016/j.tig.2018.05.008 PMID:29941292

47. Ramialison M, Waardenberg AJ, Schonrock N, Doan T, de Jong D, Bouveret R, et al. Analysis of steric effects in DamID profiling of transcription factor target genes. Genomics. 2017 Mar;109(2):75–82. https://doi.org/10.1016/j.ygeno.2017.01.006 PMID:28189763

48. Sha K, Gu SG, Pantalena-Filho LC, Goh A, Fleenor J, Blanchard D, et al. Distributed probing of chromatin structure in vivo reveals pervasive chromatin accessibility for expressed and non-expressed genes during tissue differentiation in C. elegans. BMC Genomics. 2010 Aug;11(1):465. https://doi.org/10.1186/1471-2164-11-465 PMID:20691096

49. Gómez-Saldivar G, Meister P, Askjaer P, Dam ID. DamID Analysis of Nuclear Organization in Caenorhabditis elegans. Methods Mol Biol. 2016;1411:341–58. https://doi.org/10.1007/978-1-4939-3530-7_22 PMID:27147053

50. Frøkjær-Jensen C, Davis MW, Sarov M, Taylor J, Flibotte S, LaBella M, et al. Random and targeted transgene insertion in Caenorhabditis elegans using a modified Mos1 transposon. Nat Methods. 2014 May;11(5):529–34. https://doi.org/10.1038/nmeth.2889 PMID:24820376

51. Ruijtenberg S, van den Heuvel S. G1/S Inhibitors and the SWI/SNF Complex Control Cell-Cycle Exit during Muscle Differentiation. Cell. 2015 Jul;162(2):300–13. https://doi.org/10.1016/j.cell.2015.06.013 PMID:26144318

52. Kage-Nakadai E, Imae R, Suehiro Y, Yoshina S, Hori S, Mitani S. A conditional knockout toolkit for Caenorhabditis elegans based on the Cre/loxP recombination. PLoS One. 2014 Dec;9(12):e114680. https://doi.org/10.1371/journal.pone.0114680 PMID:25474529

53. Li H. Minimap2: pairwise alignment for nucleotide sequences. Bioinformatics. 2018 Sep;34(18):3094–100. https://doi.org/10.1093/bioinformatics/bty191 PMID:29750242

54. Marshall OJ, Brand AH. damidseq_pipeline: an automated pipeline for processing DamID sequencing datasets. Bioinformatics. 2015 Oct;31(20):3371–3. https://doi.org/10.1093/bioinformatics/btv386 PMID:26112292

Supplementary information

File S1. Map of plasmid #418 in genbank format (.gb). [*hsp-16.2p::lox-P::mCherry::STOP::loxP::degron::gfp::dam::unc-54 3'UTR*]

File S2. Map of plasmid #506 in genbank format (.gb). [*hsp-16.2p::lox-P::mCherry::STOP::loxP::degron::dam::rpb-6::unc-54 3'UTR*]

Supplementary information of this article can be found online at https://jbmethods.org/jbm/article/view/362.