Chapter 11

Microscopic Analysis of Chromatin Localization and Dynamics in *C. elegans*

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Abstract

During development, the genome undergoes drastic reorganization within the nuclear space. To determine tridimensional genome folding, genome-wide techniques (damID/Hi-C) can be applied using cell populations, but these have to be calibrated using microscopy and single-cell analysis of gene positioning. Moreover, the dynamic behavior of chromatin has to be assessed on living samples. Combining fast stereotypic development with easy genetics and microscopy, the nematode *C. elegans* has become a model of choice in recent years to study changes in nuclear organization during cell fate acquisition. Here we present two complementary techniques to evaluate nuclear positioning of genes either by fluorescence in situ hybridization in fixed samples or in living worm embryos using the GFP-lacI/*lacO* chromatin-tagging system.

Key words Nuclear organization, *C. elegans*, Fluorescence in situ hybridization, Chromatin tagging, GFP-lacI/*lacO*, Microscopy

1 Introduction

Nuclear architecture has been studied in a wide range of models, including lower eukaryotes such as *S. cerevisiae* [1] and invertebrates such as *D. melanogaster*. In recent years, *Caenorhabditis elegans* has also been used, in particular to investigate the relationships between nuclear architecture and cellular differentiation [2–5]. Genome folding has been analyzed either in fixed worms and embryos using fluorescence in situ hybridization (FISH) or in live samples with the *lacO*/GFP-lacI system [2, 3, 5, 6]. Both approaches are highly complementary as each overcomes the limits of the other. The *lacO*/GFP-lacI system has the advantage of allowing the in vivo observation of chromatin position and dynamics. However, the creation of tagged chromatin loci is somehow tedious, and no more than a few loci can be observed simultaneously. In contrast, the main advantage of 3D FISH resides in the ability to detect multiple genomic segments in the same nucleus and, in combination with immunolabeling, to relate their positioning to various nuclear

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compartments. Here we present recently developed methods and reagents for both techniques and discuss the caveats, advantages, and problems associated with them.

2 Materials

2.1 3D FISH

10× NT buffer: 0.5 M Tris–HCl pH 7.5, 50 mM $MgCl_2$, 0.5 mg/mL BSA. Make 0.1 mL aliquots and store at −20 °C.

100 mM β-mercaptoethanol: 3 µL of 14.3 M β-mercaptoethanol in 0.4 mL of deionized water. Make 0.1 mL aliquots and store at −20 °C.

10× dNTP mix (*see* **Note 1**): 0.5 mM dATP, 0.5 mM dGTP, 0.5 mM dCTP, 0.2 mM dTTP. Make 20 µL aliquots and store at −20 °C.

1 mM labeled dUTP (*see* **Note 2**).

2 U/µL DNAse I.

10 U/µL *E. coli* DNA polymerase I.

0.5 M EDTA.

16 °C water bath.

PCR primers, forward and reverse, 25 µM each.

2 mM dAGC: 2 mM dATP, 2 mM dGTP, 2 mM dCTP.

1.5 mM dTTP.

5 U/µL Taq DNA polymerase.

10× Taq buffer with $MgCl_2$.

PCR cycler.

1.2 % (w/v) agarose in electrophoresis buffer (e.g., TAE 1×).

95 % EtOH, 70 % EtOH (cold).

3 M sodium acetate pH 5.2.

100 % methanol (−20 °C, in a Coplin jar).

1× PBS.

Rubber cement glue.

Deionized formamide.

Coverslips: 18×18 mm #1 thickness; 15×15 mm #1 thickness; 22×22 mm #0 thickness.

Polylysine-treated microscope slides

Metal block in dry ice, −80 °C.

4 % formaldehyde in 1× PBS.

10 mg/mL yeast tRNA stock.

20× saline sodium citrate (SSC), 2× SSC, 0.2× SSC.

50 µg/mL RNAse A in 2× SSC (freshly diluted from a 10 mg/mL stock).

2×SSC/50 % formamide.

Primary antibodies against hapten and secondary antibodies (*see* **Note 3**).

0.5 % Triton X-100 (v/v) in 1× PBS (freshly made).

0.1 N and 0.01 N HCl.

2× hybridization buffer: 20 % (w/v) dextran sulfate in 4× SSC. Make 0.5 mL aliquots and store at −20 °C.

4× SSCT: 80 µL of Tween-20 (cut pipette tip) in 400 mL of 4× SSC.

4× SSCT-BSA: 2 g of bovine serum albumin (fraction V or purer) in 50 mL of 4× SSCT.

1 µg/mL DAPI in 2×SSC.

Vectashield mounting medium (Vector Labs).

2.2 Live Chromatin Imaging

Microscope slides.

Coverslips 20×20 mm, #1.

Agarose.

Laboratory tape.

M9 buffer (KH_2PO_4 3 g/L, Na_2HPO_4 6 g/L, NaCl 5 g/L, $MgSO_4$ 1 mM).

Fine forceps.

Scalpel blade.

Mouth pipette (*see* **Note 12**).

Hourglass.

Molecular biology reagents.

- Gateway® BP Clonase® II mix.
- Gateway® LR Clonase® II mix.
- Competent DH5α.
- pDONR221 *lacO/Cb unc-119* middle vector.
- pDONRP4P1R.
- pDONRP2RP3.
- MosSCI plasmids (*see* https://sites.google.com/site/jorgensenmossci/mossci-reagents) [7–9].

C. elegans strains:

- GW396/AV696 with fluorescent lacI expression: GW396 [*baf-1p* driven somatic expression], AV696 [*pie-1p*-driven germline and early embryo expression].
- HT1593 *unc-119* mutant.

Mos insertion strain at locus of interest.

Imaging device:

Laser scanning confocal microscope/spinning disk confocal microscope.

3 Methods

3.1 3D FISH

The success of 3D FISH experiments depends crucially on the labeling of the DNA probe, i.e., the probe should be labeled uniformly. Probes should be highly specific to the target sequence and small in size to allow for efficient penetration in the sample. In this chapter, we describe the two methods of choice for probe synthesis, namely, nick translation and PCR labeling. Equally important are the pretreatment of the sample and the detection of labeled probes.

3.1.1 Probe Synthesis

The choice and synthesis of a target-specific probe is arguably the most crucial step in any 3D FISH experiment. In the case of single-copy targets, the probe should be of sufficient length to give a clear signal. For probes against the *C. elegans* genome, we routinely use fosmids that harbor 30–40 kb of sequence around the chosen genomic target. Individual fosmid clones are identified in WormBase (http://www.wormbase.org) and ordered from Source BioScience (http://www.lifesciences.sourcebioscience.com/clone-products/genomic-dna-clones/c-elegans-fosmid-library-.aspx). Fosmid DNA is labeled by nick translation. In the case of arrayed sequences (e.g., rDNA), much shorter genomic fragments (0.3–0.5 kb) can serve as template for probe synthesis, which is most conveniently done by PCR on genomic DNA. Both methods rely on the incorporation of a modified deoxynucleotide during DNA synthesis to generate labeled probes. The label is either a hapten that can be immunodetected after hybridization (digoxygenin [DIG] and dinitrophenol [DNP]) or a fluorophore. In our hands, biotinylated probes give unacceptably high background in *C. elegans* embryos, and, for this reason, we do not recommend using them.

Nick Translation

As the name implies, the nick translation technique relies on DNAse I to introduce single-strand breaks in the template DNA and on the combined 5′–3′ exonuclease and polymerase activity of DNA polymerase I to introduce labeled nucleotides starting from these pseudo priming sites. The purity of the starting material, the DNAse activity, and the incubation temperature are the most critical parameters:

1. Prepare the following mix, in this order.

DNA	1 µg
10× NT buffer	5 µL
100 mM β-mercaptoethanol	5 µL
10× dNTP mix	5 µL
1 mM labeled dUTP	1 µL (*see* **Note 4**), final concentration (20 µM)
ddH_2O	to 48.5 µL
Mix by pipetting up and down. Put tube on ice. Add the following	
DNAse I (diluted 1:15 in ddH_2O)	1 µL (*see* **Note 5**)
10 U/µL *E. coli* DNA polymerase I	0.5 µL

2. Mix well by pipetting up and down after the addition of enzymes. Do not make air bubbles. Incubate for 90 min at 16 °C.
3. Transfer the reaction on ice and analyze 1/10 of the reaction (5 µL) on a 1.2 % agarose gel (*see* **Note 6**). Include 1 kb and 100 bp ladders.
4. If the probe size is satisfactory (smear between 300 and 600 bp), proceed to the next step. If not, add 1 µL of diluted DNAse I to the reaction, return the tube at 16 °C, and incubate further for 30 min at 16 °C. Repeat **steps 3** and **4**.
5. Add 2 µL of 0.5 M EDTA to stop the reaction. Mix well.
6. *Optional.* Clean the labeled probe using ion-exchange mini spin columns (e.g., Qiagen or Zymo Research). Elute in 50 µL (*see* **Note 7**).
7. Store the labeled probe at −20 °C.

PCR Labeling

1. Prepare the following PCR reaction:

Template	200 ng of genomic DNA
Forward primer	1 µL
Reverse primer	1 µL
2 mM dAGC	5 µL
1.5 mM dT	4 µL (final concentration 120 µM)
1 mM labeled dUTP	3 µL (final concentration 60 µM)
10× Taq buffer	5 µL
ddH_2O	to 49.5 µL

2. Program the following PCR reaction: [94 °C, 3′]; [94 °C, 30″; melting temperature, 30″; 72 °C 45″] 25×; [72 °C, 10′]

3. Heat the PCR cycler to 94 °C. Incubate the probe synthesis mix for 1 min at this temperature. Add 0.5 µL of Taq DNA polymerase (2.5 U). Mix by pipetting.
4. Run the PCR program.
5. Analyze 1/20 of the PCR product (2.5 µL) on a 1.2 % agarose gel.
6. *Optional*. Clean the labeled probe using ion-exchange mini spin columns (e.g., Qiagen or Zymo Research). Elute in 50 µL (*see* **Note 7**).
7. Measure the probe concentration by spectrophotometry. Store at −20 °C.

3.1.2 3D FISH

A few points should be mentioned before detailing our 3D FISH protocol. First, since the goal of the technique is to determine the spatial localization of genomic segments, it is necessary to use fixation conditions that optimally preserve the native structure of the nucleus, i.e., to limit as much as possible the use of dehydrating agents. In the following protocol, a brief incubation in cold methanol is used to wet embryos after freeze cracking of the eggshell before fixing them in cold formaldehyde. Second, we have found it important to perform the hybridization in homemade glass chambers, so as not to compress unduly the relatively thick *C. elegans* samples (~20–30 µm). Third, as mentioned in Subheading 1, 3D FISH can be combined with the immunolabeling of cellular components. To do so, we perform the incubation with antibodies after the Triton X-100 permeabilization step, use a Cy3-labeled secondary antibody, and fix the immunocomplexes with formaldehyde before proceeding with the FISH protocol. Finally, it should be noted that unlike for hybridization to mammalian DNA, it is not necessary to quench the repetitive sequences when performing FISH on *C. elegans* DNA because these make up a much lower proportion of the genome in this organism (the most abundant repeat element in *C. elegans*, the 439 bp long CE000087, covers only 1.32 % of the genome [10]).

Preparation of the Probe Mix

1. Mix the following in an Eppendorf tube, *in this order*.

NT probe 1	*x*µL (typically corresponding to 400 ng of probe)
NT probe 2	*x*µL (typically corresponding to 400 ng of probe)
...	
10 mg/mL tRNA	1 µL (*see* **Note 8**)
Deionized water	to 100 µL
3 M NaOAC pH 5.2	10 µL
95 % EtOH (cold)	400 µL

2. Vortex vigorously. Incubate at −20 °C for at least 2 h.
3. Centrifuge at 13,000 × *g* for 20 min at 4 °C.
4. Decant supernatant. Wash pellet with 0.5 mL of cold 70 % EtOH. Air-dry on the bench for 2–3 min.
5. Add 20 µL of deionized formamide to the pellet (the final probe concentration is 20 ng/µL each). Incubate at 37 °C for 20 min. Pipette up and down to resuspend pellet.

Fixation and Pretreatment of Embryos

1. Isolate embryos according to standard protocols, e.g., by the "bleaching" method.
2. Deposit 12 µL of concentrated embryos in the center of a poly-lysine-treated slide. Cover gently with an 18 × 18 mm coverslip.
3. Working at low magnification under the stereomicroscope, adsorb liquid using a filter paper until the embryos are slightly compressed between the slide and the coverslip.
4. Place the slide on a metal block in dry ice. Incubate at −80 °C for at least 60 min.
5. Using a razor blade, pop up the coverslip (i.e., "cracking" the eggshell). Proceed immediately to next step.
6. Immediately fix embryos in cold 100 % methanol, 2 min at −20 °C.
7. Rinse slide(s) 1 min in 1× PBS at 4 °C (*see* **Note 9**).
8. Transfer the slide(s) to precooled (4 °C) 4 % formaldehyde in 1× PBS. Fix for 10 min at room temperature.
9. Wash slides in 1× PBS, two times for 2 min at room temperature.
10. Incubate for 5 min in 0.5 % Triton X-100 in 1× PBS.
11. Wash two times for 2 min in 1× PBS.
12. Rinse once in 0.01 N HCl.
13. Incubate for 2 min in 0.1 N HCl at room temperature.
14. Wash once with 1× PBS, 3 min at room temperature. Wash once with 2× SSC, 3 min at room temperature.
15. Treat with 50 µg/mL RNAse A in 2× SSC, 45 min at 37 °C. Perform this step by overlaying the sample with 0.5 mL of the RNAse solution and incubating in a humidified chamber.
16. Wash once with 2× SSC, 2 min at room temperature.
17. Incubate in 2× SSC/50 % formamide for at least 2 h at room temperature.

Hybridization and Post-hybridization Washes

1. Dilute the probe to a concentration of 2–5 ng/µL in 100 % deionized formamide. Add an equal volume of 2× hybridization buffer. 25 µL of probe solution is needed per sample (*see* **Note 10**).

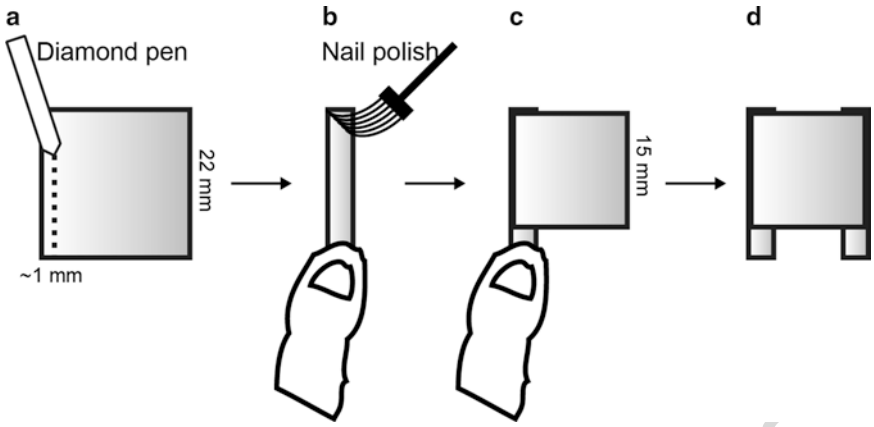

Fig. 1 Making homemade hybridization chambers. (**a**) Using a diamond pen, cut a ~1 mm strip from the side of a 22 mm × 22 mm coverslip (*thickness #0*). (**b**) Apply nail polish on one side of the glass strip. (**c**) Immediately glue the glass strip to the side of a 15 mm × 15 mm coverslip (thickness #1). (**d**) Leave to dry and gently break off the protruding pieces of glass

2. Mix well by pipetting and vortexing. Spin 20 s in a tabletop centrifuge. Keep the probe at room temperature.
3. Take a slide out of the 2× SSC/50 % formamide solution. Gently wipe off excess liquid on either side of the sample using a soft tissue.
4. Prepare a glass chamber: glue with nail polish two small strips of glass of thickness #0 (width of ~1 mm) to the opposite sides of a 15 × 15mm coverslip (*see* Fig. 1).
5. Pipette 25 µL of probe solution on a glass chamber.
6. Using forceps, gently deposit the chamber on the slide, probe facing towards the sample. Let the probe solution spread to the edges of the chamber.
7. Seal the chamber with rubber cement glue. Let dry completely at room temperature.
8. Repeat **steps 3**–7 for each slide.
9. Pre-hybridize the slides overnight at 37 °C ~~overnight~~.
10. Denature the probe and target DNA simultaneously by placing the slide for 5 min on a heating block set at 76 °C.
11. Incubate at 37 °C for 2–3 days.
12. Fill four Coplin jars with 2× SSC. Preheat three of those at 37 °C. Fill two other Coplin jars with 0.2× SSC and preheat at 55 °C.
13. Gently remove the rubber cement glue around the chamber. Do not remove chamber.
14. Place the slide(s) in a Coplin jar filled with 2× SSC at room temperature. The chamber should fall off. If it does not, pull it gently alongside the slide.

15. Wash for 3×5 min in 2× SSC at 37 °C.
16. Wash for 2×5 min in 0.2× SSC at 55 °C.

Detection

1. If only fluorophore-labeled probes were used, go directly to **step 17**.
2. Set aside a 1 mL aliquot of 4× SSCT-BSA for the dilution of antibodies.
3. Transfer the slide to 4× SSCT-BSA. Incubate for 20 min at room temperature.
4. Dilute the primary antibodies in 4× SSCT-BSA. Use only those antibodies that are needed (*see* **Note 3**).
5. Remove the slide from the blocking solution. Gently wipe off excess liquid on either side of the sample.
6. Pipette 60 µL of primary antibody solution close to the sample. Overlay with an 18×18mm coverslip and make sure that the antibody solution covers the sample.
7. Incubate for 2 h at room temperature in a humid chamber, protected from light.
8. Place the slide in a Coplin jar filled with 4× SSCT at room temperature.
9. Wash for 3× 5 min in 4× SSCT at room temperature.
10. Dilute the secondary antibodies in 4× SSCT-BSA. Use only those antibodies that are needed.
11. Centrifuge the secondary antibody solution for 2 min at 13,000×*g*. Transfer the supernatant to another microtube.
12. Remove a slide from the washing solution. Gently wipe off excess liquid on either side of the sample.
13. Pipette 60 µL of secondary antibody solution close to the sample. Overlay with an 18×18 mm and make sure that the antibody solution covers the sample.
14. Incubate for 1 h at room temperature in a humid chamber, protected from light.
15. Place the slide in a Coplin jar filled with 4× SSCT at room temperature.
16. Wash for 2× 5 min in 4× SSCT at room temperature.
17. Wash for 1× 5 min in 4× SSC at room temperature.
18. Gently pipette 100 µL of DAPI solution (final concentration is 1 µg/mL) on the sample. Incubate for 2 min at room temperature, protected from light.
19. Rinse in 2× SSC and mount with Vectashield. Seal with nail polish.

The protocols presented here have been applied successfully to perform 3D DNA FISH on embryos up to the 150- to 200-cell stage, after which time the signal decreases from the outside to the inside of the embryo due to poorer probe and antibody penetration. Samples are usually imaged on a laser scanning confocal microscope using a 63× plan apochromat oil objective (numerical aperture of 1.4), with optical sections taken at intervals of 300–700 nm. We have observed that some fosmid probes (about 1 in 5) give a signal that consists of several dots of variable intensities throughout the nucleus, especially when direct-labeled with fluorophores. The reason for this high background remains unknown. In any case, the simplest solution to this problem is to replace the bad fosmid with a neighboring or even overlapping one. Typical 3D DNA FISH results are shown in Fig. 2. In this experiment, we used the centrosomes as extranuclear reference points in order to be able to compare gene positioning in the same blastomere from

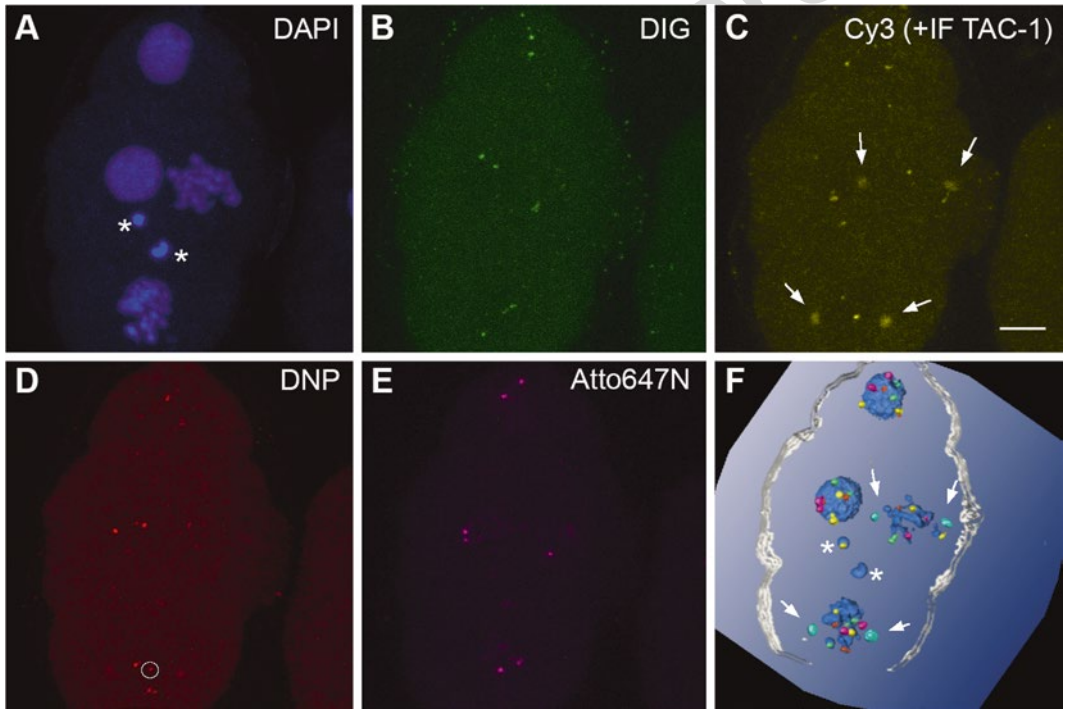

Fig. 2 Analysis of gene positioning in early *C. elegans* embryo using immuno-DNA FISH. Raw data (maximal projections, **a**–**e**) and 3D reconstruction (**f**) of a 4-cell stage embryo that was hybridized to the following probes. (**b**) Fosmid WRM0634bG05 (chr. X) labeled with DIG and detected with mouse anti-DIG and anti-mouse FITC. (**c**) Fosmid WRM0619aE04 (chr. V) labeled with Cy3, TAC-1 protein labeled with mouse anti-TAC-1 and anti-mouse Cy3 prior to FISH. (**d**) Fosmid WRM0628cE09 (chr. II) labeled with DNP and detected with rabbit anti-DNP and anti-rabbit Texas Red. (**e**) Fosmid WRM0623bE05 (chr. III) labeled with Atto647N. Remnants of polar bodies (*asterisks*) hybridize poorly due to high level of DNA condensation. Centrosomes are labeled weakly but clearly (*white arrows*). Bleeding of strong Cy3 signals in the Texas Red channel is sometimes observed (*circled* in (**d**))

different embryos. As expected, the background is somewhat higher when using hapten-labeled probe (DIG and DNP), due to the use of primary/secondary antibodies in the detection protocol, but signals are nonetheless clear.

3.2 Live Chromatin Imaging

In *C. elegans*, a number of laboratories have successfully validated the *lacO*/lacI system for tagging loci in vivo [2, 3, 5, 11–13]. The technique is a two-step process. The first step involves the expression of a fusion between a DNA-binding protein, a fluorescent protein, and a nuclear localization signal. Second, *lacO* repeats are inserted into the genome either as high-copy injected plasmid arrays, as low-copy bombarded transgenes, or as a single-copy insertion. Strains expressing different lacI fusions can be obtained from the laboratories which created them (*see* below). We therefore focus on the creation of *lacO* insertions, in particular at a given locus using a modified MosSCI transposon-mediated homologous recombination procedure [7]. In the last part, we describe how to mount live embryos for microscopy and the imaging setups adapted for *C. elegans*.

3.2.1 Strains for Expression of GFP-lacI/lacI-GFP

The DNA-binding Lac repressor is expressed as a fusion with green fluorescent protein (GFP). Expression levels of the protein have to be kept low, as overexpression elevates the background fluorescence, enhances nonspecific binding, and can cause slow-growth/sick animals. Two strains expressing GFP-lacI from integrated arrays have been published to date. The first one is based on injected integrated arrays, where GFP-lacI is expressed from the housekeeping promoter *baf-1* (GW396 [2]). The second is based on a bombarded construct in where GFP-lacI is transcriptionally regulated by a *pie-1* promoter (AV696 [5]). In the strain GW396, GFP-lacI is visible from about the 20-cell stage to adulthood due to germline silencing (note that this array has a *vit-5::GFP* intestinal marker expressed from the late L4/adulthood, which hinders GFP-lacI observation). In the AV696 strain, fluorescent signal decreases rapidly at the beginning of embryogenesis, but is useful to locate chromatin in the germline or in early embryos. The two types of expression constructs cannot be used in conjunction due to germline silencing in *trans* of the *pie-1* promoter construct [14]. Both constructs do not contain *lacO* sites in the plasmids used to create them and therefore do not create a GFP-lacI spot.

3.2.2 Creation of lacO-Tagged Transgenes and Insertion of lacO into the Genome

To allow visualization of a given transgene or locus, a number of binding sites for the bacterial repressor have to be integrated into the genome. Most of the *C. elegans* published arrays obtained by gonadal injection have been created using plasmids that contain a single *lacO* site (the 17 bp consensus sequence recognized by lacI; e.g., all the Fire library plasmids contain this sequence as a single copy). Due to the high-plasmid-copy number in the injected arrays

(several hundreds of copies), they are readily detected by GFP-lacI and create a visible spot, usually at the nuclear periphery linked to their silencing [2, 4]. Smaller transgenes (in the range of 10–50 copies) bound by lacI can be created by co-bombarding plasmids of interest with *lacO* repeats (256 repeat, pSR1 [2, 15]). Although the *lacO* repeats do not get integrated each time, this method leads to about 50 % co-integration rate, with transgenes visible when GFP-lacI is expressed in *trans*. These low-copy transgenes are not subject to silencing and display usually a random localization in early embryos [2, 4]. Finally, single targeted genomic insertions of *lacO* arrays can be achieved using MosSCI and derivatives [6, 7]. As this method is new, a detailed description is given below.

As few as 24 lacI binding sites (*lacO*) are sufficient to allow the formation of a visible spot, although the signal-to-noise ratio depends on the expression level of the fluorescently tagged binding protein lacI. We use 256 *lacO* repeats (about 12 kb in size); however, the actual number of sites which get integrated is likely to be lower. Plasmids with binding site repeats are intrinsically recombinogenic in bacteria. It is therefore highly recommended to use recA strains (DH5α/XL1 blue) or recB recJ (SURE) for amplification/cloning. When defreezing these strains, it is good practice to isolate single colonies and test the length of *lacO* repeats as the repeat stretch has a tendency to shrink in size.

To insert *lacO* repeats at a site of interest, one needs a *Mos* insertion located next to the site of interest. Due to the resolution of optical microscopes, "near" means in the next 20–40 kb [16]. Many *Mos* insertions (about 13,000) have been created by the NemaGENETAG project (http://elegans.imbb.forth.gr/nemagenetag/). These are available as invalidated or validated insertions. All available insertions can be browsed at http://pbil.univ-lyon1.fr/segalat/data/index.php and ordered at http://ums3421.univ-lyon1.fr/spip.php?article14. This is the most cost-effective manner to create *lacO*-tagged loci, as compared to ZFN/TALEN methods (Transcription Activator-Like Effector Nuclease [17]), in which the nuclease is engineered to create a double-strand break at a given sequence, providing a template for homologous recombination. MosSCI however requires a *Mos* insertion to be available in the region of interest. However, given that 20 kb genomic distance cannot be resolved using light microscopy, several kilobases can separate the region of interest and the *Mos* insertion. [AU1]

Insertion of the *lacO* repeats is achieved using Mos Single-Copy Insertion (MosSCI [7]) and requires homology between the insertion site and the plasmid that serves as a template for homologous recombination. To efficiently achieve the creation of templates with homology surrounding *lacO* repeats, we designed a triple-plasmid Gateway system with a middle *lacO/Cb unc-119* plasmid. This is used to create a plasmid where the *lacO* repeats are flanked with homology on both sides. [AU2]

1. Prepare the *C. elegans* recipient strain by crossing it to an *unc-119(ed3)* mutant and selecting for unc worms homozygous for the *Mos* insertion of interest. At this point, it is good to cross in the transgene for the expression of GFP-lacI to have a visual confirmation of *lacO* integration later (*see* above for strain names).

[AU3]

2. Design primers to amplify left and right sequences located 5′ and 3′ of the *Mos* insertion site (Fig. 3a). A size of 1.5 kb is sufficient for efficient recombination [18]. Primers are designed for Gateway BP cloning.

   Use following 5′ extensions for the following primers from a to d (in 5′ → 3′ direction):

   (a) GGGG ACA ACT TTG TAT AGA AAA GTT G—locus-specific sequence

   (b) GGGG AC TGC TTT TTT GTA CAA ACT TG—locus-specific sequence (reverse complement)

   (c) GGGG ACA GCT TTC TTG TAC AAA GTG G—locus-specific sequence

   (d) GGGG AC AAC TTT GTA TAA TAA AGT TG—locus-specific sequence (reverse complement)

   To minimize amplification errors, we use Phusion polymerase (NEB) according to the manufacturer's instruction with the following cycling conditions: [98 °C, 30″]; [98 °C, 10″; 45 °C, 30″; 72 °C 30″/kb] 5×; [98 °C, 10″; 50 °C, 30″; 72 °C, 30″/kb] 20×; 72 °C, 10′ 12 °C ∞.

   Test amplicon length and perform BP cloning according to the manufacturer's manual (Invitrogen), except that it is preferable to transform and plate at least half of the cloning reaction to get a sufficient number of colonies. In our hands, BP cloning has been highly efficient, with at least seven out of eight colonies tested positive for the desired insertion.

3. Perform the Gateway LR reaction with the 5′, middle [see note above about *lacO* repeats plasmid], and 3′ clones (Fig. 3b). Transform at least half of the LR reaction to get a sufficient number of colonies. Test the plasmids by PCR/miniprep.

4. Test the length of *lacO* sites on the destination vector. These should be about 10 kb in size to make a visible spot in vivo. Normally the *lacO* repeats length does not change drastically during the LR recombination, as recombination uses the Clonase mix and transformation is carried in DH5α strains.

5. Carry on MosSCI according to the original protocol [7, 8] (Fig. 3c). In our hands, integration at certain loci was more difficult to achieve than at others. The indirect, heat-shock procedure was preferred in such cases.

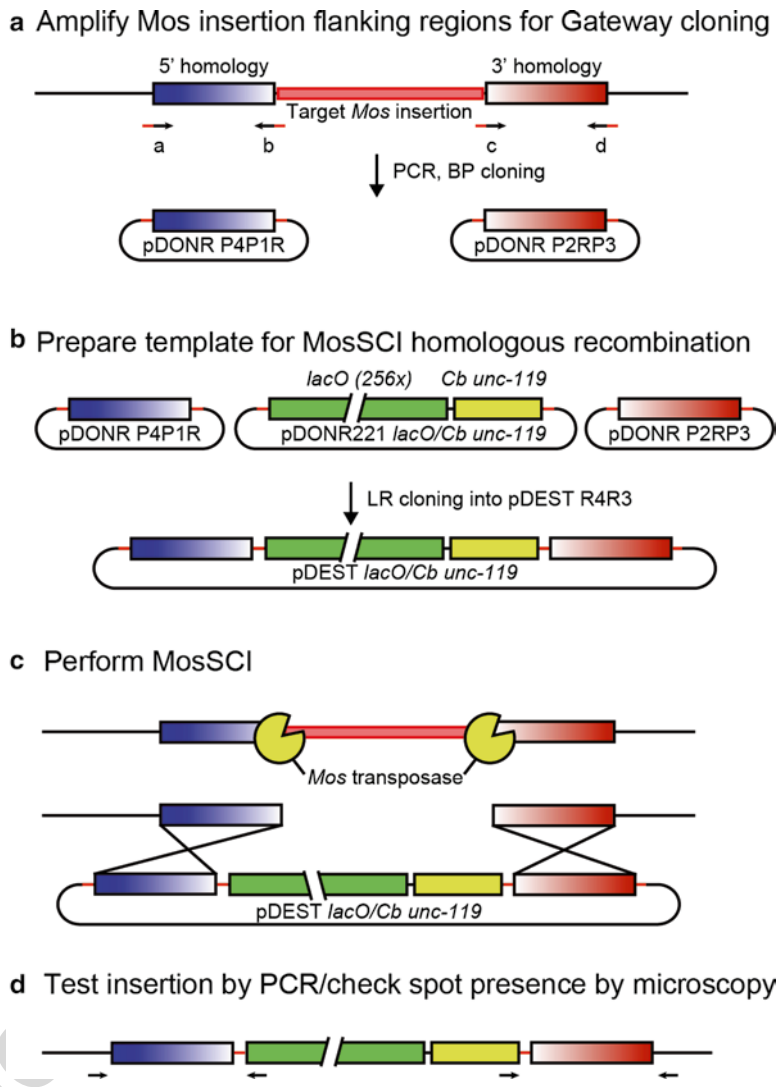

Fig. 3 Method for insertion of *lacO* repeats at a given *Mos* insertion site. (**a**) Regions flanking the *Mos* insertions are amplified by PCR using primers a, b, c, and d with Gateway-specific overhangs (*red*, *see* sequence in the text). Amplified homology stretch is recombined in the Gateway 5′ and 3′ vectors using BP. (**b**) The template for recombination is created by LR recombination of the vectors created in (**a**) with the middle *lacO/Cb unc-119*. (**c**) *Mos*-mediated single-copy integration is carried out in the strain carrying the *Mos* insertion of interest in an *unc-119(ed3)* background, which leads to the integration of the *lacO/Cb unc-119* template at the *Mos* locus. (**d**) Insertion is tested by PCR using primers located in the *lacO/Cb unc-119* insertion and outside of the homology stretch

6. Check insertion by PCR using primers outside of the homology region and in the inserted transgene (Fig. 3d). Also, check for the presence of a visible spot under the microscope. During the homologous recombination procedure, some *lacO* repeats might get lost, which therefore makes the GFP-lacI spot difficult or impossible to see.

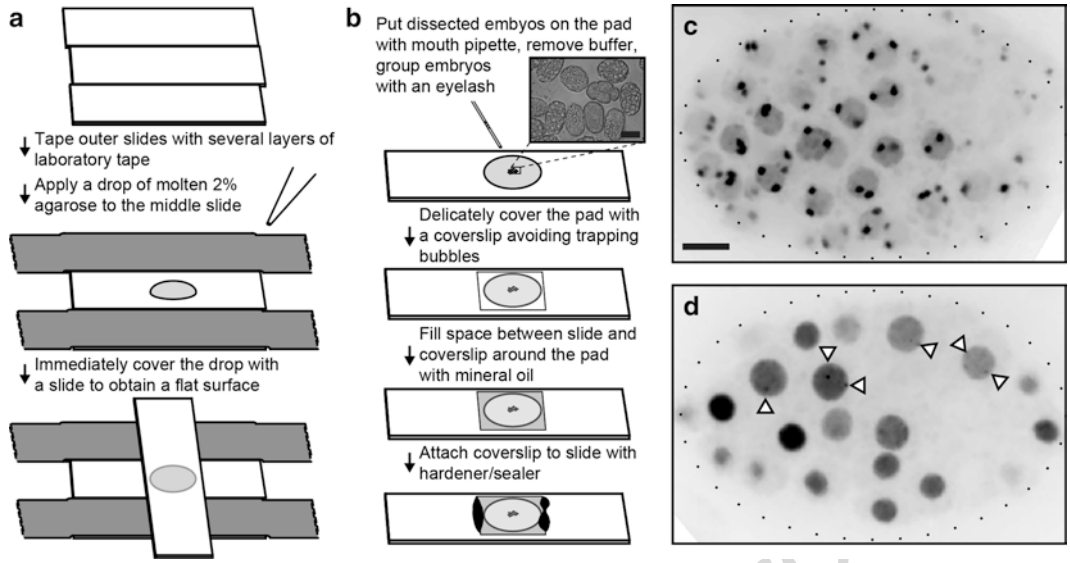

Fig. 4 Imaging live embryos carrying *lacO* inserts. (**a**) Preparation of the agarose pad, using three microscope slides. The outer two slides are taped to the bench using thick laboratory tape. Additional layers of tape will determine the thickness of the agarose pad. A drop of molten 2 % agarose is placed in the center of the middle slide and immediately covered with a perpendicular slide, creating a flat pad. (**b**) Embryos dissected in an hourglass are transferred to the agarose pad using a mouth pipette. Excess buffer is removed with the pipette, and embryos are grouped with an eyelash (bar = 20 μm). After covering the embryos with a coverslip, the space around the pad is filled with injection oil, and the coverslip is sealed to the slide using Vaseline/lanoline/paraffin or Vaseline alone. (**c**) Axial z projection of a stack of optical slices of an embryo expressing GFP-lacI from an integrated large array with *lacO* (GW76, *gwIs4[baf-1::GFP-lacI; myo-3::RFP]X* [2]). Two large spots corresponding to the homolog chromosomes are visible in each nucleus (bar = 5 μm). (**d**) Axial z projection of a stack of optical slices of an embryo expressing GFP-lacI from an integrated large array without *lacO* and an insertion of *lacO* repeats created using the technique described in Fig. 1 (GW392, *gwIs39[baf-1::GFP-lacI; vit-5::GFP]III gwSi13[lacO/Cb unc-119@ttTi9115]V* [4]). The GFP-lacI-expressing array is not visible as it carries no *lacO* sites. The single-copy insertion forms very small spots (*arrows*)

3.2.3 Preparing Worms and Embryos for Imaging

Preparing Agarose Pads for Microscopy

1. Prepare the molding bench (Fig. 4a).

   Put three microscope slides next to each other on a flat surface. Stick both outer slides with tape to the surface, putting thick laboratory tape on top of the slides. The thickness of the pad will depend on the thickness of the tape glued on the outer slides. Put two to three additional layers of tape on the slides to increase the thickness of the pad.

2. Prepare 2 % agarose in water and melt at 95 °C. Agarose can be aliquoted in 0.5–1 mL tubes for future use and stored at room temperature. Remelting of aliquoted agarose is achieved by putting the solution at 95 °C for 5 min followed by brief vortexing. The molten agarose can be kept longer at 70 °C instead of 95 °C. At high temperatures, agarose hydrolyzes and does not harden anymore. To image (moving) worms instead of embryos, add 0.5 μL 10 % NaN_3 to the agarose to

inhibit movement. Alternatively, anesthetizing worms with 400 mM ethanol has been recently shown to deliver similar results [19].

3. Prepare the agarose pad (Fig. 4a). Put a drop (50–100 µL) of molten agarose on a slide placed in the middle of the two taped slides. Immediately place a second slide, perpendicularly to the first one, creating a flat surface. This can be kept for several hours if the upper slide is not removed (otherwise the pad dries out and becomes unusable).

Preparing Embryos for Live Imaging

1. Pick gravid adults with fine forceps, and move them to an hourglass filled with M9 (*see* **Note 11**). Try to avoid taking too much bacteria as *E. coli* will stick to embryos once dissected and usually has high autofluorescence. If needed, move the adults with a platinum loop to bacteria-free plates prior to putting them in the M9.
2. Cut adults in two in the middle of the worm (approximately where the vulva is). Squeeze delicately the head and tail parts to release the embryos from the adult carcasses.
3. Using a mouth pipette, aspirate the embryos (*see* **Note 12**).
4. Transfer the embryos to the agarose pad (Fig. 4b). Make sure to hold the tip of the mouth pipette vertically during the transfer as failure to do so will lead to dripping of embryos out of the capillary. Once over the agarose pad, blow in the mouth pipette to expel the embryos, allow them to settle on the agarose (20 s), and aspirate delicately excess M9 with the mouth pipette. The M9 layer should be at a minimum height, so that embryos do not move anymore when an eyelash is put on the pad.
5. Group embryos together using an eyelash tip (a flexible eyelash fixed on a tip/toothpick with nail polish). This is very helpful for finding the embryos under the microscope and minimizes the search time at high magnification.
6. Put a coverslip on top of the pad/embryos, making sure to avoid trapping too many bubbles, in particular close to the embryos as they diffract light. The coverslip should be laid at an angle on one side of the pad and then slowly lowered on the embryos, pushing away bubbles.
7. For longer acquisition times, it is recommended to minimize evaporation (which would flatten the pad, damage embryos, and shift focus). We use injection oil (mineral oil, Sigma M5904) to hinder water exchange between the pad and the exterior. Injection oil has the advantage to allow oxygenation, and embryos can develop and hatch if kept overnight in such a chamber. Put enough oil to fill the gap between the slide and the coverslip around the agarose pad.

8. In order to avoid movement of embryos and sliding of the coverslip/pad, glue the coverslip to the slide using a 1:1:1 mixture of Vaseline/lanoline/paraffin (on two sides only to avoid impairing gas exchanges). Alternatively, Vaseline can be used alone but is less solid. Both types of glue have to be melted.

3.2.4 Imaging Living Embryos

As for any live imaging, it is good practice to evaluate whether the development of the worms was not disturbed by the laser exposure. This is especially important when studying chromatin dynamics, as animals will experience repeated illumination over an extended period of time. Evaluating whether the movements observed correspond to normal behavior or to DNA repair-linked activities is important. An easy way to check this is to compare illuminated and control embryos at approximately the same stage at time of fluorescent imaging and follow their development over time using DIC imaging.

Imaging *lacO*-tagged chromatin from embryos is challenging: embryos are relatively thick (about 20 μm) and diffract light; the *lacO*/GFP-lacI spot can be very small and highly dynamic; moreover, the fluorescent signal is low as overexpression of lacI is deleterious for the animals and *C. elegans* embryos are highly sensitive to focused light. Several imaging systems were assayed and the best system for this application was a spinning disk confocal microscope. Confocality is essential as for single-copy *lacO* repeats, the observed structure is small (Fig. 4d); out-of-focus haze would make it invisible. Two systems can achieve confocal images: point scanning and spinning disk confocal microscopes. In the first system a focused laser beam is moving over the sample at high speed; emitted light is filtered for out-of-focus photons by passing through a pinhole and acquired by a photomultiplier. In these systems, maximum laser power as well as laser damage is concentrated on a single point. An alternative solution which minimizes excitation/damage is spinning disk confocal devices. In spinning microscopy, the laser beam is split in hundreds of focused sub-beams, rotating on the sample at high speed. The laser power on each point as well as the time spent by the laser on each point of the sample is minimized but repeated several hundred times per second. Emitted light is filtered through the pinholes, and photons are acquired using a highly sensitive CCD camera. The whole field of view can be acquired in as little as 30 ms; acquisition speed is limited by the frame reading rate of the camera. Using a correct combination of lenses/objective/camera, sampling will meet the Nyquist rate (about 90 nm/pixel). The thickness of the embryos (about 20 μm) and the small size of *lacO* transgenes make it very difficult to obtain images of entire embryos while keeping them alive (in the *z*-axis). Emitted light from objects far from the objective is diffracted by the structures above. Hence, when acquiring image stacks of embryos, we limit our acquisition to the 10 μm closer to the

objective if embryos have to be imaged repeatedly for a longer period of time. Alternatively, if only one time point is to be acquired, we apply high laser power to be able to record light emitted from nuclei located far away from the objective.

Typical images acquired with a spinning disk confocal (TILL Photonics) of embryos carrying two different types of *lacO*-tagged chromatin are shown in Fig. 4. Large arrays obtained by gonadal injection and chromosomal integration (about 300–500 copies of the injected plasmids, 3–5 Mb in size) create large heterochromatic structures, which are located at the nuclear periphery in relation with their epigenetic silencing (Fig. 4c [4]). In Fig. 4d, an embryo with 256 *lacO* repeats integrated as a single copy using the technique described above is shown (Subheading 3.2.2). In each nucleus, the GFP-lacI spot is smaller and emerges from background nuclear fluorescence created by unbound GFP-lacI. Finally, it has to be noted that chromatin inside the nucleus is dynamic; hence precise positioning of the GFP-lacI/*lacO* spots necessitates a fast acquisition device. Typically, imaging should not be more than 100 ms per optical slice.

4 Notes

1. Due to the AT richness of the *C. elegans* genome (66 %), we increase the final concentration of unlabeled dTTP in the nick translation reaction to 20 μM. The molar ratio of labeled dUTP to unlabeled dTTP is 1:1 (compared to 2:1 or higher in most protocols).
2. Our preferred labeled nucleotides for a 4-color 3D DNA FISH experiment are DIG-dUTP, Cy3-dUTP, DNP-dUTP, and Atto647N-dUTP. We have also used successfully FITC-dUTP and Cy5-dUTP. However, labeling with Atto594-dUTP and Atto488-dUTP proved to be inefficient.
3. We use the following primary antibodies at a 1:200 dilution to detect hapten-labeled probes: mouse anti-digoxygenin (Jackson ImmunoResearch) and rabbit anti-DNP (Sigma). The secondary antibodies (1:400 dilution) are highly adsorbed FITC-conjugated goat anti-mouse and Texas Red-conjugated goat anti-rabbit, both from Jackson ImmunoResearch.
4. While the quantity of labeled dUTP given here works well in most cases, we found it had to be increased to 3 μL for efficient labeling with FITC-dUTP.
5. The optimal dilution of DNAse I has to be determined empirically and will result in the generation of fragments of 300–600 bp in length after a 90 min incubation. The activity is mainly influenced by the purity of the template DNA. Initial experiments should include a range of dilutions, from 1:500 to 1:10.

6. DIG-labeled fragments migrate slower than unlabeled ones of similar length. DNP- and Cy3-labeled probes are poorly stained by DNA-intercalating agents (e.g., ethidium bromide, SYBR Green). Note that the reaction can be stored at –20 °C at this point. If the reaction needs to be resumed after storage, a fresh aliquot of *E. coli* DNA polymerase I should be added to the reaction.
7. The cleaning of labeled probes (e.g., on ion-exchange spin columns) is optional. However, we have found that performing this step leads to an improvement in signal-to-noise ratio in experiments involving *C. elegans*, and we recommend doing it.
8. We use tRNA as a carrier during probe precipitation instead of ssDNA, which sticks to the eggshell and thus gives undesirable background upon DAPI staining.
9. We have found that slides have to be transferred from cold methanol (–20 °C) to cold PBS and then cold formaldehyde (4 °C) in order to minimize the loss of embryos. By doing so, we also found that subsequent incubations and washes could be performed in Coplin jars without significant loss of embryos from the slide.
10. The final probe concentration is 1–2.5 ng/μL. In our hands, this range of concentration gives excellent results. The *C. elegans* probes are repeat poor and can therefore be used at a lower concentration than the ones that are used in FISH experiments on mammalian samples.
11. In our hands, GFP fluorescence is always higher when worms have been grown at temperature above 20 °C. This is likely due to GFP variants optimized for mammalian expression at 37 °C, as we also observed that fluorescence is higher after heat shock at 34 °C.
12. Mouth pipettes are made using capillaries. (a) A 10 μL pipette is heated in the flame of an ethanol burner. (b) Once the glass is soft, remove it from the flame and quickly pull apart the ends. (c) Break the ends apart to create a pipette with an end with a diameter of ~40 μm. (d) Place the pipette in a mouth pipette aspirator (Sigma A5177 or building plans are available upon request).

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