Chapter 22

Using RNA Tags for Multicolor Live Imaging of Chromatin Loci and Transcription in Drosophila Embryos

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Abstract

Elucidating the biological implications of higher order chromatin architectures in animal development requires simultaneous, quantitative measurements of chromatin dynamics and transcriptional activity in living specimen. Here we describe a multicolor labeling and live imaging approach in embryos of the fruit fly *Drosophila melanogaster*. The method allows simultaneous measurement of movements of specific loci and their transcriptional activity for developmental genes, enabling new approaches to probe the interaction between 3D chromatin architecture and regulation of gene expression in development.

Key words Quantitative live imaging, *Drosophila* embryos, Transcription, 3D genome architecture

1 Introduction

There is growing evidence for a regulatory role of the 3D architecture of chromatin for the control of gene expression during development and disease processes [1–3]. Ligation or thin sectioning-based genomic approaches provide snapshots of the global DNA connectivity map [4, 5]. However, the dynamics of the 3D chromatin architecture at the single-cell level has been difficult to assess due to fixed and pooled samples applied in such genomic assays. Here we present a multicolor labeling and live imaging method to visualize the movement of specific DNA loci in fly embryos. In particular, we combine molecular systems based on nascent RNA visualization and systems based on direct tagging of DNA loci to simultaneously measure the transcriptional activity of key developmental genes and their cis-regulatory elements using fluorescence microscopy. This method allows measurement and analysis of 3D chromatin dynamics and gene activity in real time and with single live-cell resolution.

Using CRISPR-Cas9 technology, endogenous genes are edited with RNA stem-loop cassettes, which were pioneered in single-celled organisms to study RNA transcription, splicing, and

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transport [6–8]. Two orthogonal systems (MS2 and PP7) have been adopted for *Drosophila* [9–11]. Nascent mRNAs transcribed from the tagged loci carry stem-loops at their 5′ ends, preferably intronic regions. These stem-loops are recognized by corresponding genetically encoded coupling or coat proteins fused to various fluorescent proteins, enabling visualization of the physical locations and transcriptional activity of the tagged loci. Furthermore, we introduced a DNA labeling approach based on the bacteria DNA partitioning system (parABS). Here, a *parS* sequence from *Burkholderia* was incorporated into the designated loci [12–14] and maternally supplied ParB proteins then bound to the *parS* sequence and aggregated to form diffraction-limited spots.

In this protocol, we provide details regarding the construction of CRISPR-edited flies, embryo preparation and mounting, choice of fluorescent protein combinations for multicolor live imaging, and optimized conditions of point-scanning confocal microscopy for acquisition of high-quality 3D time-lapse images for localization analysis. In particular, we discuss the relevant controls required to assess the experimental errors associated with the measured distance between the tagged loci.

2 Materials

2.1 Fly Lines

1. Maternal Cas9 lines [15, 16] (*see* **Note 1**).
2. Maternal phiC31 integrase lines [17] (*see* **Note 2**).
3. Maternal lines that contain three fluorescent protein fusions for three-color live imaging [18] (*see* **Note 3**).

2.2 Labeling Endogenous Loci

1. Plasmids for CRISPR-based Drosophila genome editing [15] (*see* **Note 4**).
2. Plasmids for recombination-mediated cassette exchange (RMCE) [19, 20].
3. Plasmids for stem-loop series [7] (*see* **Note 5**).
4. Plasmids for parS-based genome labeling [12, 18] (*see* **Note 6**).

2.3 Genome Editing and Recombination

1. Embryo collection cages.
2. Agar plates: 1% Agar in 20% Welch's concord grape juice.
3. Mesh wells for embryo collection.
4. Halocarbon 200.
5. 50% Commercial bleach: 4.1% Sodium hypochlorite solution.
6. Double-sided tapes (Scotch, permanent).
7. Cover glass (22 × 50 mm).

8. Drying chamber (15 × 10 × 5 cm) filled with Drierite desiccant (3 cm in depth).
9. Femtotip II microinjection needles (Eppendorf).
10. FemtoJet pump (Eppendorf).
11. Microinjection station.
12. Humidity chamber.
13. Drosophila food vials.
14. Fluorescence stereoscope for phenotyping.

2.4 Embryo Live Imaging

1. Embryo collection cages.
2. Agar plates: 1% Agar in 20% Welch's concord grape juice.
3. Ultrathin air-permeable membrane (Lumox film 25 um, Sarstedt Inc.).
4. 3D printed membrane holders [21].
5. Dissecting needles.
6. Heptane glue (*see* **Note 7**).
7. Halocarbon 27.
8. Cover glass (18 × 18 mm, No. 1.5).
9. Confocal microscope with multiple laser lines and high-sensitivity photon detectors.
10. Three-color-coated TetraSpec beads (200 nm).
11. Autofluorescent plastic slide (Chroma) for flat-field adjustment.

3 Methods

We provide detailed protocols for generating fly lines that carry multiple-edited genomic loci. Crossing males from these lines with the three-color females will produce embryos for multicolor live imaging. We advocate strict controls during sample preparations and live imaging procedures in order to obtain high-quality and reproducible image data for quantitative analysis.

3.1 Editing of Endogenous Genes to Contain MS2, PP7, and parS Tags

Cas9-mediated homologous recombination is used to integrate RNA stem-loop sequences or parS sites at the target gene loci. To enhance the flexibility of the in vivo design, an intermediate step using recombination-mediated cassette exchange (RMCE) is adopted (Fig. 1a).

3.1.1 Cas9-Mediated Knock-In

1. Select a guide RNA (gRNA) target site that contains a protospacer adjacent motif (PAM, 5′-NGG-3′) [15]. The PAM site should be as close to the targeted locus as possible. Two gRNA

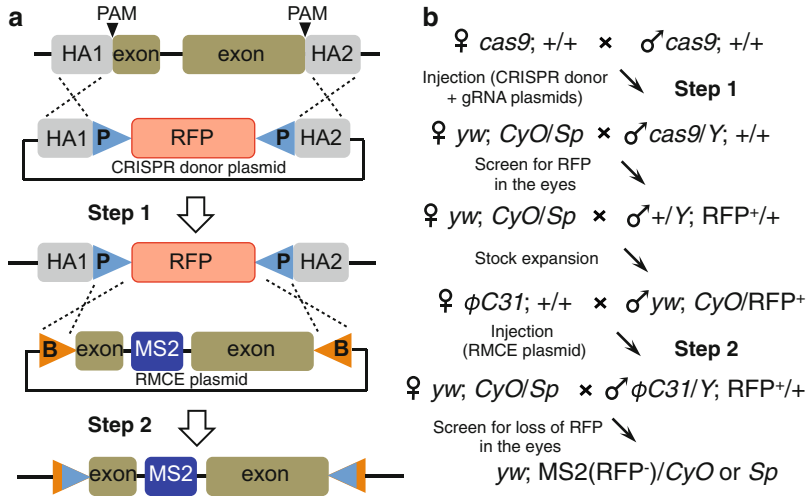

Fig. 1 Genomic integration of live imaging tags through CRISPR-Cas9-based genome editing and attB/attP cassette exchange. (**a**) A two-step approach to insert MS2 stem-loops in the intron of a target gene. In the first step, the intron and the adjacent exons are replaced by an eye-expressing RFP marker flanked by two attP sites (P) through CRISPR-mediated homologous recombination. PAM, protospacer adjacent motif. HA, homologous arm. In the second step, the deleted genomic sequence with an MS2 tag in the intron is inserted between two attB (B) sites and introduced back to the genome through attB/attP recombinations. The same method also applies to other imaging tags (PP7 or parS). (**b**) Crossing scheme to generate transgenic flies through the two-step integration approach. A target gene on the second chromosome is shown as an example. Appropriate balancers should be used for target genes on the other chromosomes (*see* **Note 12**)

target sites can be used to delete up to 20 kb genomic region if genomic modifications, such as deletions and point mutations, are desired in the following experiments. Depending on the locus of interest, the deleted region may include coding sequences and/or cis-regulatory elements.

2. Sequence the gRNA recognition sites from the target genome to avoid point mutations.
3. Generate plasmids that express the gRNAs [22] (*see* **Note 8**).
4. Clone the flanking homologous regions into the donor plasmid, in which a phenotypic selection marker flanked with two attP sites is located between the two homologous arms (*see* **Note 9**). The attP sites are designed for introducing stem-loops or parS tags through RMCE.
5. Set up embryo collection cages with germline-supplied Cas9 females and males carrying the target genome. Embryos collected from these cages are used for fly transformation.
6. Follow Subheading 3.2 to generate transgenic flies with the targeted deletion and integrated attP sites by co-injecting the gRNA-expressing plasmid (or two plasmids if two gRNA target sites are selected) and the homologous donor plasmid to embryo germline.

3.1.2 Integration of MS2 Stem-Loops, PP7 Stem-Loops, or parS Tags Through RMCE

1. Construct the RMCE plasmid that contains the designated stem-loops or parS sequence flanked by two attB sites (*see* **Note 10**). If a deletion is generated from the CRISPR step, the deleted genomic region should also be included in the RMCE plasmid.
2. Set up embryo collection cages with germline-supplied phiC31 females and males that carry the Cas9-mediated attP knock-in. Embryos collected from these cages are used for fly transformation. Follow Subheading 3.2 to generate transgenic flies with the integrated imaging tags. Negative selection, i.e., loss of the marker introduced with the Cas9-mediated knock-in, is used to identify transformants.

3.2 Generation of Transgenic Fly Lines

Transgenic fly lines are generated through germline injections. In-home injections are recommended to facilitate fly maintenance and speed up experiments. Different loci are generated separately and genetically combined in cis or trans to create embryos that carry multiple-edited loci.

3.2.1 Embryo Collection and Mounting

1. Collect ~300 virgin females from the maternal Cas9 or integrase lines and put them together in an embryo collection bottle with ~100 males that carry the targeted genomic loci. Multiple collection bottles may be set up to increase embryo yield.
2. 0–1-h-old embryos are collected on agar plates. 100–200 embryos are enough for a 1-h injection session.
3. To dissolve chorion, add 50% bleach onto the plates for 1 min. Swirl gently every 20 s. Pour the de-chorionated embryos into a plastic mesh well. Rinse with tap water for 1 min.
4. Transfer embryos onto a 60 × 30 × 10 mm 1% agar block and align them perpendicular to the edge of the block. The anterior tips of the embryos should face to the edge. Leave a space of 2 mm between the embryo tips and the edge of the agar block. The adjacent embryos should be separated by ~1 mm. An experienced injector is able to align 40 embryos in about 6 min.
5. Cover the long side of a piece of 50 mm cover glass with double-sided tape. Adhere the aligned embryos to the tape on the cover glass. The embryos' posterior tips should point to the edge of the coverslip after transfer.
6. Dry the embryos in the desiccant chamber. Drying time varies between 0 and 5 min depending on seasons and weather conditions (*see* **Note 11**).
7. Take out the coverslip from the desiccant chamber and cover the embryos with Halocarbon 200.
8. Repeat **steps 4**–**7** for 20 min or until all embryos are mounted.

3.2.2 Germline Injection

1. Load 4 μL of DNA solution to the Femtotip II needle and mount the needle to the microinjection station.
2. Load the embryo slide on the microinjection station.
3. Adjust the position of the needle to make sure that the embryos and the tip of the needle are on the same focal plane.
4. Gently push the needle into the germplasm. The embryo does not leak if appropriate drying time is applied.
5. Adjust the compensation pressure on the FemtoJet to avoid absorption of germplasm into the needle. For a new needle, 50 hPa is desirable. Compensation pressure needs to be adjusted during injection when the needle is worn or partially clogged.
6. Adjust the injection pressure to five times as high as the compensation pressure and inject.
7. After all the embryos on the cover glass are injected, move the cover glass into a humidity chamber saturated with water vapor. Larvae are expected to hatch after 48 h at 18 °C or after 24 h at 25 °C.
8. Pick the larvae using a dissecting needle and transfer them into regular Drosophila food vials (20–30 larvae per vial). Avoid halocarbon during the transfer.
9. When the adults hatch, cross them with appropriate balancer lines (*see* **Note 12**). In the next generation, screen for the gain or loss of selection markers (Fig. 2b, also *see* **Note 13**). PCR genotyping is optional to confirm the edited region.
10. Two edited loci can be recombined in cis following standard Drosophila genetics protocols. Screening for recombinants should be performed 3 days after hatching if eye markers are used.

3.3 Embryo Preparation for Live Imaging

1. Set up crosses to get triple-heterozygous females that supply all three fluorescent proteins (MCP-3xtagBFP2, PCP-3xmKate2, and ParB-eGFP).
2. Collect ~100 three-color virgin females and put them in an embryo-collecting cup with ~50 males that carry the edited loci (Fig. 2a).
3. Set up a sample holder with a piece of air-permeable membrane as described in [9]. Add 50 μL of heptane glue on at the center of the membrane. The glue should be completely dried when the embryos are mounted.
4. Collect embryos at desired age on agar plates.
5. Cut a piece of double-sided tape (2 cm long) and push it against the surface of the agar plates to adhere embryos on it.

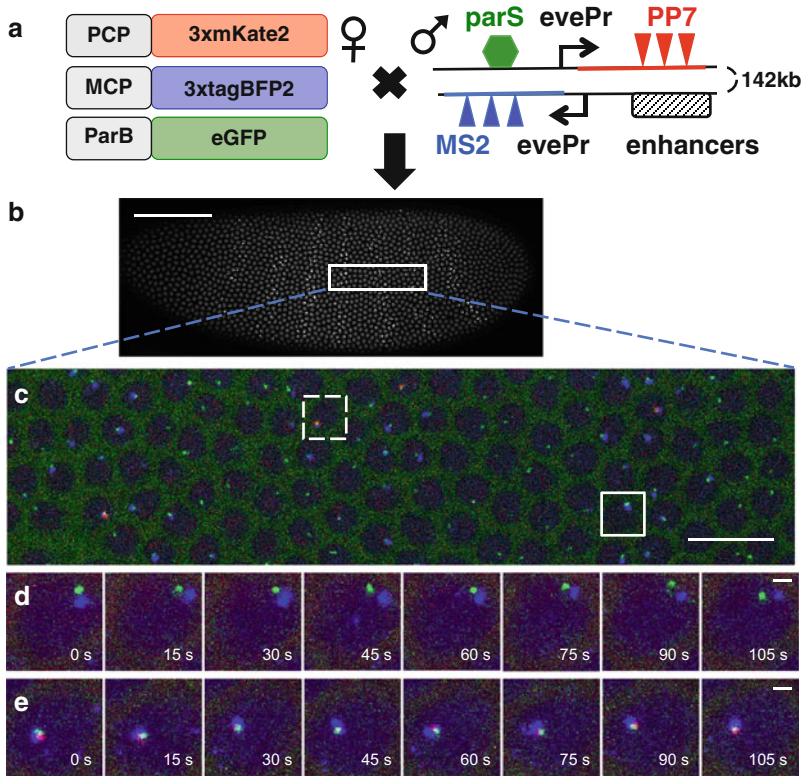

Fig. 2 Three-color live imaging of interactions between tagged genomic loci in *Drosophila* embryos. (**a**) Male flies carrying genomic loci labeled with MS2, PP7, and parS tags are crossed with females containing three fluorescent protein fusions that recognize the specific tags. In this example, we designed a synthetic system to visualize the long-range interaction between the *even-skipped* (*eve*) enhancers and a transgene reporter located 142 kb upstream to the *eve* locus. The endogenous *eve* gene is labeled with MS2 stem-loops, while the reporter transgene is labeled with PP7 stem-loops and parS tag. Physical locations and transcriptional activity of the tagged *eve* and reporter loci are measured in the embryos generated from this genetic cross. (**b**) Snapshot of a representative embryo from three-color live imaging; blue channel for the MS2 signal is shown; anterior is to the left. (**c**) Overlay image of the three channels of the region marked by the white box in (**b**). Note that the blue spots (MS2) mark the locations of the *eve* locus (via tagged nascent RNA), green spots (parS) mark the locations of the reporter transgene, and red signal (PP7) indicates transcription activity of the reporter transgene. (**d**) A time series of eight snapshots following the nucleus marked with the solid white box in (**c**). Note that the blue and the green signals are physically separated and the red signal is not present during the time course. (**e**) A time series of eight snapshots following the nucleus marked within the dashed white box as in (**c**). Note the presence of red signals and the overlap of the blue and the green spots. All images are *z* (apical-basal axis) projections from 25 optical sections covering 8 μm. Scale bars: 100 μm in (**b**), 10 μm in (**c**), and 1 μm in (**d**) and (**e**)

6. Flip the double-sided tape. Use a dissecting needle to gently push each embryo from the direction perpendicular to its anterior-posterior axis, so that chorion sticks to the tape and breaks to expose the inside of the embryo. Prevent the embryo vitelline from touching the tape.

7. Mount the de-chorionated embryos on the air-permeable membrane with the designated dorsal-ventral orientation. The orientation should be adjusted before the embryos touch the glue (*see* **Note 14**). Finish embryo mounting in 8 min at 40% humidity and 25 °C. 20–30 embryos are expected to be mounted.
8. Add 50 μL of halocarbon 27 on the embryos and cover them with an 18 × 18 mm cover glass (No. 1.5). Avoid any shearing. The embryos should keep their dorsal-ventral orientation.

3.4 Imaging of Embryos

Here we provide protocols and microscope setups for the Leica SP5 point-scanning confocal system. The protocols are also suitable for other multicolor confocal microscopes (e.g., Zeiss, Nikon). Image examples are shown in Fig. 2. Three laser lines are used: a diode laser for 405 nm, an argon laser for 488 nm, and a HeNe for 591 nm. The powers are set at 0.4 μW, 1.1 μW, and 0.5 μW, respectively. Light intensities at the sample are estimated to be 13, 36, and 17 $\mu W/cm^2$, respectively.

1. Start imaging when the embryo develops to the desired stage. Find the embryos with bright-field illumination using a 20× objective and save their XY locations.
2. Acquire images through a high-magnification (63×), high-NA (1.44) oil-immersion objective. Use HyD photon counters to provide high sensitivity and a broad dynamic range. Set voxel size to 107 × 107 × 330 nm. For cellular blastoderm, image a z-stack of 8 μm that covers the entire nuclei at the embryo periphery.
3. Scan at 700 Hz and accumulate three scans per line. Two sequential scans are used, alternating with each line, 405 nm and 591 nm scan simultaneously, followed by the 488 nm scan.
4. Once the acquisition is done, take a full embryo image at the midsagittal plane using a 20× objective in order to register anterior-posterior positions.
5. Take a flat-field image using a Chroma autofluorescent plastic slide. Reduce power to avoid saturation. The flat-field image is used to control uneven illumination in the image field of view.

3.5 Correction of Errors Associated with Fluorescent Spot Distance Measurements

1. Measure three-color TetraSpec beads at different laser powers to obtain images with intensities matching the dynamic range of the acquired MS2-, PP7-, or parS-system-tagged spots.
2. Measure a control fly line in which blue, green, and red spots are physically co-localized. We use a reporter line with alternating MS2 and PP7 stem-loops. Cross males from this line with females carrying NLS-MCP-3xtagBFP2, MCP-eGFP, and PCP-mCherry (or PCP-mKate2). Image the embryos with the same microscope settings.

3. Localization errors are estimated from the distributions of the distance between the co-localized spots measured from fluorescent beads and live embryo control constructs. The variance of distance distribution from these controls can be subtracted from the mean squared distances between two spots obtained from a time series of measurement (e.g., Fig. 2d, e).

3.6 Image Analysis

Custom MATLAB code is available upon request. The code includes nuclear registration, spot segmentation and tracking, chromatic aberration correction, and MSD analysis. Detailed descriptions can be found in [18].

4 Notes

1. In these lines, Cas9 is materially expressed from a nos-Cas9 or vas-Cas9 source integrated at designated genomic locations. See genome editing resource at Bloomington Drosophila Stock Center (BDSC) for more information (https://bdsc.indiana.edu/stocks/genome_editing/crispr_cas9.html). In this protocol, we engineered the second chromosome as an example and used BDSC #51324, in which a vas-Cas9 transgene is inserted at the third chromosome and marked with a body color marker (*yellow*, *y*).
2. We used BDSC #34770 as the maternal phiC31 integrase source. These female flies provide integrase activity in their germlines, which induces attB/attP recombination to incorporate transgenes. For more information about the selection of integrase lines, *see* https://bdsc.indiana.edu/stocks/phic31/index.html.
3. eGFP is fused to the N-terminus of the Burkholderia parB protein. Due to the slow turnover of ParB proteins at the parS site, the highly photostable eGFP fusion ensures long-time visualization of the parS-tagged loci. MCP is fused with a 3xtagBFP2. A nuclear localization signal (NLS) is added to the fusion, which leads to strong nuclear enrichment of the fusion protein. This increased nuclear background helps to overcome the relatively low photostability of blue fluorescent proteins and allows continuous detection of transcription spots across the blastoderm stage. PCP is fused with 3xmKate2 to provide good spectral separation from the green channel. An NLS is added to increase nuclear concentration of the fusion protein. NLS-PCP-3xmKate2 is enriched in the cytoplasm until 10 min before gastrulation. Three transgenic lines (nos-NLS-MCP-3xtagBFP2, nos-NLS-PCP-3xmKate2, and vas-ParB-eGFP) were generated separately through germline injections. A homozygously combined stock (PCP-3xmKate2; vas-ParB-eGFP) is maintained and available upon request.

4. See flyCRISPR (http://flycrispr.molbio.wisc.edu/) and Addgene (http://www.addgene.org/crispr/oconnor-giles/) for details about CRISPR designs. We used Addgene 51434 for generating CRIPSR donor plasmid for homology-directed repair and Addgene 45946 for generating plasmids expressing guide RNAs.
5. The stems and/or the linkers in the step loop series may contain binding sites for tissue or temporal specific transcription factors; thus tagging the gene with stem-loop sequences may influence its expression pattern. A quantitative comparison between the expression pattern of the edited locus and the endogenous gene is recommended. In the case where undesired ectopic expression occurs, stem or linker sequences should be modified.
6. parS sequence from *Burkholderia cenocepacia* (J2315, chr3:3440-3821, GB: AM747722) is used for genome labeling. The method is adapted from reference [12]. See Addgene (https://www.addgene.org/Kerstin_Bystricky/) for more information about Bystricky group plasmids.
7. To create heptane glue, cut ~100 cm of permanent Scotch double-sided tape and put it into a 20 mL scintillation vial. Add 15 mL of heptane. Rock the vial on a horizontal shaker (100 rpm) for 24 h. Transfer the heptane into a 15 mL Corning tube and centrifuge at 1000 × *g* for 10 min. Take the supernatant and store at 4 °C.
8. It is recommended to test the cutting efficiency of the two target gRNA plasmids before homologous donor injection.
9. Homologous arms from the target genome should be sequenced and used for constructing the integration donor plasmid. Length of homologous arms can range from 0.5 to 1.5 kb.
10. Presence of more than 12 repeats of MS2 or PP7 stem-loops in the 5′UTR of the tested genes (hb, Kr, kni, eve, and run) caused gene inactivation as seen by the lack of phenotypic rescue. In order to create viable tagged gene constructs able to rescue a null mutant, it is recommended that the stem-loops are integrated in the intron or in the 3′UTR.
11. A drying test before formal injections is recommended. An empirical indication of appropriate drying time is that about 20% embryos leak when the needle shoves into the germplasm.
12. Balancer lines contain chromosomes with multiple inversions, which prevent viable meiotic recombinations. This is particularly important for tracking and maintaining transgenes that lack visible markers. See BDSC information about balancers (https://bdsc.indiana.edu/stocks/balancers/index.html).

13. We recommend fluorescent eye markers (e.g., RFP or GFP). These are most clearly identified in a *white* (*w*) mutant background. If a *w* + background is used, adult flies should be screened 3 days after emergence for fluorescence. Ommatidia near the equator are the most conspicuous spots of fluorescent signals.
14. Adjust dorsal-ventral orientation using the dissecting needle. Gently rotate the embryos on the needle tip with the help of the broken chorion.

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