

Multi-labeling live imaging to quantitate gene expression dynamics during *Drosophila* embryonic development

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Running head

Quantitative live imaging of gene expression

Abstract

Transcription in developing metazoans is inherently stochastic, involving transient and dynamic interactions among transcriptional machinery. A fundamental challenge with traditional techniques, including fixed-tissue protein and RNA staining, is the lack of temporal resolution. Quantitating kinetic changes in transcription can elucidate underlying mechanisms of interaction among regulatory modules. In this protocol, we describe the successful implementation of a combination of MS2/MCP and PP7/PCP systems in living *Drosophila* embryos to further our understanding of transcriptional dynamics during development. Our technique can be extended to visualize transcriptional activities of multiple genes or alleles simultaneously, characterize allele-specific expression of a target gene, and quantitatively analyze RNA polymerase II activity in a single-cell resolution.

Key words: quantitative live imaging; transcription; *Drosophila* embryos; MS2; PP7; development

1. Introduction

The decades of prior research in *Drosophila* embryos have yielded extensive knowledge of well-identified enhancers and gene expression patterns, making *Drosophila* an ideal model organism to study the role of temporal control on tissue morphogenesis and pattern formation (1, 2). Since development is a dynamically evolving process, achieving a high temporal resolution is

necessary to perceive subtle changes in the kinetics of transcriptional activity in real time. The MS2/MS2 coat protein (MCP) and PP7/PP7 coat protein (PCP) systems provide a platform to quantitatively visualize and characterize the dynamics of gene expression upon perturbation of the genome in living tissues. The multi-labeling technique utilizes MS2 and PP7 tandem repeat sequences and RNA phage-originated coat proteins that specifically bind to the MS2 and PP7 RNA stem loops (3, 4). Typically, MS2 and PP7 repeat sequences are inserted to the 5' UTR, 3' UTR, or intronic regions of the gene of interest. Upon transcription, MS2 and PP7 sequences form stem loop structures, and nascent RNA transcripts are visualized as fluorescent puncta, as maternally provided MCP or PCP molecules fused with a fluorescent protein bind to the MS2 or PP7 stem loops, respectively (Figure 1A) (5–8).

The modularity and orthogonality of this technique present new opportunities to examine transcriptional dynamics in different organisms. For example, we can specifically tag a target gene on one allele with MS2, tag the same or different target gene on the other allele with PP7, and visualize the interactions of the two alleles within a single nucleus (Figure 1B) (9–12). This approach has been used to compare allele-specific expression of a gene, and to compare transcription kinetics of a wildtype and mutant allele in the same nucleus (13). In the case of a single enhancer activating two promoters, multi-labeling can be utilized to distinguish between the two promoters' transcriptional activities by tagging each promoter with either MS2 or PP7 (Figure 1C) (14, 15). Moreover, we can extend the MS2 and PP7 imaging system to determine the parameters that affect the rate of RNA Polymerase II (Pol II) elongation and their downstream effects on transcription kinetics (Figure 1D) (16, 17). Here, we describe a detailed protocol to generate transgenic and CRISPRed fly lines with MS2- and PP7-tagged target genes,

mount *Drosophila* embryos, and quantitatively image and analyze transcriptional dynamics using the multi-labeling live imaging system. The technique outlined in this chapter can be applied to study transcription during key developmental processes, including tissue morphogenesis.

2. Materials

2.1 Fly lines

1. *Nanos promoter (nos) > MCP-GFP, nos > mCherry-PCP, His2Av-eBFP2 (9)* or fly lines that can maternally express MCP and PCP fused with a fluorescent protein.
2. Fly stock carrying the MS2- and/or PP7-tagged reporter gene.
3. Fly stock containing the endogenous gene of interest tagged with MS2 and/or PP7 through genome editing.

2.2 Embryo collection

1. Embryo collection cages
2. Apple juice agar plates
3. Dry yeast
4. Paintbrush
5. Empty petri dish

2.3 Embryo mounting

1. Microscope slide and insert
2. Semi-permeable membrane
3. Heptane glue
4. Empty petri dish
5. 50% bleach solution

6. Deionized (DI) water
7. Halocarbon oil 27
8. 18x18 mm coverslip
9. Stereoscope

2.4 Imaging

1. Confocal microscope with 40x or 63x oil immersion objective
2. Immersion oil

2.5 Image analysis

1. Fiji image processing software (**18**)
2. MATLAB (MathWorks)

3. Methods

3.1 Generation of fly lines

1. For transgenic lines, design a construct in which a specific enhancer drives transcription of a reporter gene tagged with MS2 and/or PP7. Use an appropriate targeting plasmid for site-specific or random integration of the transgene into the fly genome (**19**).
2. For visualizing transcriptional activity of endogenous genes, CRISPR-mediated genome editing is commonly used (**20**). A guide RNA vector and a donor vector containing MS2 and/or PP7 sequences and homology arms are generated (**21**). The gRNA vector and donor vector are injected to embryos carrying the Cas9 protein.
3. Generated fly lines are screened and cultured for future experiments.

3.2 Setting up the cage

We use *nos > MCP-GFP*, *nos > mCherry-PCP*, *His2Av-eBFP2* flies to visualize simultaneous transcriptional activity of MS2 and PP7 in either one or two alleles. The His2Av-eBFP2 protein allows for nuclei tracking in each nuclear cycle, while the maternally deposited MCP-GFP and PCP-mCherry proteins are used to visualize gene expression. For visualizing the MS2- and PP7-tagged gene expression in the same allele (i.e., Figures 1C and D), the homozygous male flies carrying the tagged gene of interest are crossed with virgin female flies containing His2Av-eBFP2, MCP-GFP, and PCP-mCherry.

Below, we have detailed the genetic crosses necessary to visualize transcriptional activity of two alleles, each tagged with either MS2 or PP7 stem loops, in a single nucleus (i.e., Figure 1B).

1. Virgin female flies containing His2Av-eBFP2, MCP-GFP, and PCP-mCherry proteins are mated with homozygous male flies carrying the gene of interest tagged with MS2 (or PP7) (Figure 2A).
2. Progeny from the first cross contain one copy of His2Av-eBFP2, MCP-GFP, PCP-mCherry, and one copy of the gene of interest tagged with MS2 (Figure 2B). The cage is set up using the virgin female progeny from the first cross and homozygous males carrying the PP7-tagged target gene (Figure 2B). It is crucial that the females, not males, from the first cross are used here to ensure the maternal deposition of MCP-GFP and PCP-mCherry. If a PP7-tagged stock was used in the first cross, the cage would then require homozygous males carrying the gene tagged with MS2. See Note 1.
3. The resulting progeny from the cage will be either *nos > MCP-GFP*, *nos > mCherry-PCP*, *His2Av-eBFP2 / enhancer>PP7* or *enhancer>MS2 / enhancer>PP7* (Figure 2C). The embryos containing *enhancer>MS2 / enhancer>PP7* allele will exhibit both MS2

and PP7 fluorescence and allow us to visualize both alleles' activities simultaneously (Figure 3E).

It is recommended that the cage is set up with at least 35 virgin females and 25 males to ensure enough embryos are laid in the allotted time frame (Figure 3A). The cage should be set 2-3 days prior to imaging and kept at 22-23°C. All the embryos that are collected for subsequent imaging are laid on apple juice agar plates. Add extra yeast granules to the cage plate and change the plate once every day to maintain healthy flies. Imaging the embryos ~10 days after setting up the cage may increase chances of unhealthy or unfertilized eggs. See Note 2.

3.3 Embryo mounting

1. Depending on the developmental stage to be imaged, change the cage plate to a new one and let the flies lay eggs for a desired duration. After the necessary time has elapsed, change the plate again and retain the old plate (with embryos laid) for mounting (Figure 3B). See Note 2.
2. Cut a piece of semi-permeable membrane and fit it between the insert and microscope slide (Figure 3C). Add a drop of heptane glue, spread it around the membrane, and let it sit for at least 5 minutes or until completely dry.
3. Under the stereoscope, collect the embryos off the apple juice agar plate using a fine-tipped paintbrush and gently place them on a small piece of paper towel on an empty petri dish.
4. Dechorionate embryos by soaking them in 50% bleach solution for about 2 min. See Note 3.

5. Dry the excess bleach using small pieces of paper towel.
6. Wash the embryos with DI water and soak up the remaining water with small paper towel pieces. Do this step twice. See Note 4.
7. Using tweezers, place the paper towel piece containing the embryos into a clean empty petri dish filled with DI water. Place the paper towel piece in the water until all the dechorionated embryos are in the water.
8. Using the paintbrush, gently scoop an embryo off the water's surface and place it on the glue-coated membrane. Under the stereoscope, make sure the embryo is in the correct orientation to visualize the target gene, and that the region to be imaged is facing upwards (for an upright microscope). For easier imaging, align the embryos in a grid fashion (e.g., 5x5) (Figure 3C). See Note 5.
9. Once all the embryos are mounted, add three drops of halocarbon oil: one drop in the top left corner, one drop in the top right corner, and one drop in the middle at the bottom of the slide. Carefully, place the coverslip over the embryos. The oil should spread and completely cover the embryos. See Note 6.

3.4 Live imaging of gene of interest

This setup involves using Zeiss LSM800 confocal laser scanning microscope (Figure 3D). Images are acquired with a Plan-Apochromat 40x or 63x oil objective using a 405, 488, and 561 lasers to visualize His2Av-eBFP2, MCP-GFP, and PCP-mCherry, respectively, at a time resolution of 12s/frame to 25s/frame depending on the setting. For each frame, a z-stack is acquired to cover the entire mono-nuclear layer. Ensure that the temperature in the room remains constant throughout the imaging experiment. See Note 7.

1. Once the correct objective is in place, add a small drop of immersion oil on the objective lens. Place the microscope slide upside down for an upright microscope.
2. Using brightfield light or a fluorescent filter, locate all of the mounted embryos that are in the correct developmental stage and mark their positions.
3. Using the 405-laser channel (to visualize His2Av-eBFP2), observe the size and density of the nuclei to confirm the nuclear cycle of the embryo.
4. Set the laser powers to detect MS2 and PP7 signals to reasonable percentages. Use the range indicator feature to avoid saturating the detector or photobleaching. See Note 8.
5. Once a desired embryo is chosen, begin acquiring z-stacks. Typically, we image from the top to the bottom of the mono-nuclear layer to ensure that all of the MS2 and PP7 signals are captured. Continue imaging until the end of the developmental time of interest has been reached. See Note 9.
6. If a partial view of the embryo was captured, zoom out or use the tile-mode setting to image the entire embryo and verify its orientation. This will ensure that no spatial information is lost during imaging.
7. If there are no visible MS2 or PP7 signals, see Note 10.

3.5 Image processing

This step involves creating maximum intensity projection images from the raw image files and generating a single movie spanning the entirety of the imaging experiment. This requires installing an image processing software. The following steps are completed in Fiji.

1. Open all the experiment files using Fiji.

2. Adjust the color histogram (Image>Adjust>Brightness/Contrast) and channel color (Image>Color>Channels Tool), as necessary for visualization purposes. See Note 11.
3. Create a maximum intensity projection of each image using the z-projection feature (Image>Stacks>Z Project – Maximum intensity). This will create a new file with the maximum signal intensity across all the z-stacks for each time frame. Do this for each experiment file.
4. If multiple experiment files were generated per embryo, concatenate the maximum intensity projection files (Image>Stacks>Tools>Concatenate). This feature will merge all the experiment files into one movie. Snapshots from a representative movie are shown in Figure 3E and 4A.

3.6 Image analysis

All the image processing methods and analyses described below are implemented in MATLAB (R2018b, MathWorks) (22).

3.6.1 Nuclei segmentation and tracking

1. The His2Av-eBFP2 channel is used to segment the nuclei using watershed segmentation. Use Gaussian filtering to minimize the background noise and then convert to binary images. Manually correct frames, as necessary (Figure 3F).
2. Obtain the number of nuclei that were segmented and assign x and y coordinates to the center of mass of each nucleus for tracking. A single nucleus that exhibits minimal movement across the segmented frames (within the same nuclear cycle) is deemed to be the same nucleus in each time frame.

3.6.2 Signal extraction

This step explains the process by which the MS2 and PP7 signal data are extracted from segmented nuclei.

1. To remove the background signal, the MS2 or PP7 signal in a given nucleus is adjusted by subtracting the median value across all the time frames. Since the MS2 or PP7 puncta are much brighter than the background MCP and PCP nuclear signal, the median value of the nucleus represents an average background MCP and PCP signal.
2. To determine the MS2 or PP7 signal intensity in a given nucleus, average the top two or three pixels with the highest intensities (depending on the zoom). This is done to account for variability in signal intensities.

3.6.3 Plot generation

The fluorescence intensity at a given time frame in each nucleus is used as a proxy to measure instantaneous amplitude of a given transcript. Representative traces of fluorescence intensities for both MS2- and PP7-tagged genes can be extracted and plotted on a single graph (Figure 3G). Six randomly selected nuclei in Figure 4A display variable signal intensities throughout the nuclear cycle. Despite the stochasticity observed in individual traces (Figure 4B), the average trajectories can be obtained and utilized to characterize kinetic behavior over time. The cumulative mRNA output of a nucleus is computed by integrating a nucleus's trajectory of fluorescence intensity over time (Figure 5A). After extracting single nucleus data, the distributions of different parameters such as mRNA output or maximum fluorescence intensity can be visualized in the form of a boxplot, with individual data points overlaid on the plot. (Figure 5B).

Multi-labeling live imaging provides a platform to analyze dynamic transcriptional activity. For example, it has been shown that a single shared enhancer can coactivate two

tagged target promoters simultaneously, resulting in regions of coordinate bursting (Figure 5C) **(14)**. Additionally, a single gene of interest can be tagged with both MS2 and PP7, with one tag in 5' UTR and the other in 3' UTR of the gene (Figure 1D). As Pol II begins transcription, MS2 signal is observed. Once Pol II transcribes the entire gene, PP7 signal becomes visible (Figure 5D). This time delay, Δt , between the MS2 and PP7 signals can be used as a proxy for the time needed to transcribe the entire gene. Since the length of the gene is known, the rate at which Pol II traverses along the gene (i.e., the elongation rate of Pol II) can be computed by dividing the length of the gene by the time difference between the two signals **(16)**.

In addition to single nucleus resolution, live imaging provides embryo-wide spatial information. The heatmap in Figure 5E is an example of the *snail* (*sna*) expression pattern generated using individual nuclear output. It highlights the distribution of *sna* RNA production – with the nuclei producing higher levels of mRNA in the center and nuclei with lower production levels at the borders, but also reveals significant nuclei-to-nuclei variability. Figure 5F shows each nucleus's transcriptional activity in time, sorted from the early activating nuclei to the late activating ones. This type of analysis offers a depiction of nuclear activation kinetics and can aid in understanding the spatial arrangement of active nuclei.

Moreover, the technique detailed in this protocol and the cross described in Figure 2 offer the possibility to study the effects of allele-specific genetic manipulations and perturbations in real time **(13)**. As seen in Figure 6A, the endogenous *even-skipped* (*eve*)

alleles, each tagged with either MS2 or PP7, exhibit similar activation behaviors. When the stripe 1 enhancer is deleted from the MS2-tagged *eve* allele, however, *eve* is no longer expressed in the stripe 1 domain (Figure 6B). Interestingly, by overlaying the MS2 and PP7 signals, we can also visualize an unexpected expansion of the *eve* stripe 2 expression when the stripe 1 enhancer is deleted. The multi-labeling imaging technique presented in this protocol can be extended to visualize and analyze transcriptional activity of genes of interest during various developmental processes.

4. Notes

1. If *nos* > *MCP-GFP*, *nos* > *mCherry-PCP*, *His2Av-eBFP2* flies are first crossed with the PP7-containing flies rather than the MS2 ones, then the progeny to be imaged would contain maternal PP7 and paternal MS2 alleles. The lineage of the MS2 and PP7 alleles, whether maternal or paternal, should not affect the transcriptional activities of the alleles (10).
2. If there are insufficient embryos laid during the desired developmental time window, consider adding more virgin females and males to the cage and wait 1-2 more days before imaging. If the issue persists despite having >50 females in the cage, placing the cage in an incubator at 25°C may help improve embryo yield.

Additional tips to increase the number of embryos laid:

- a. Keep the cage in a dark room.
- b. Warm the cage plate to room temperature before changing the plate.
- c. Use newly hatched virgins and males.

3. If embryos are not dechorionated after 2 minutes, you may increase the time to 3 minutes. Prepare a fresh 50% bleach solution if this issue persists. It is important to avoid overbleaching, as this may cause the embryos to become too soft or unhealthy. In this case, decrease the bleaching time.
4. During the washing step, use the edges of the paper towel and be careful not to accidentally soak up the embryos. Add just enough water to wet all the embryos but not so much as to cause them to slide off the paper towel.
5. An embryo may look unhealthy and experience extreme nuclear fallout or asynchronous mitoses due to internal damage or high pressure. An embryo may also undergo an additional nuclear cycle prior to gastrulation (nuclear cycle 15). Avoid moving the embryos too much after they are in the correct orientation to prevent these issues.
6. Place the microscope slide coverslip from one side, rather than directly setting it in the middle of the membrane to avoid disrupting the embryos' orientations. If the embryos shift too much after placing the coverslip, you may have added too much oil while mounting. The embryos should not move after setting the microscope slide cover. If the oil does not cover the entire slide, you may need to use more oil.
7. Make sure to keep the developing embryos at a constant temperature as development can be affected by increase or decrease in temperature. A temperature chamber can be used to maintain the temperature of the sample.
8. If the signal seems weak and/or faint, increase the laser power to a suitable percentage. Photobleaching may occur if the laser power is too high.
9. During imaging, the embryo may shift enough such that the uppermost MS2 or PP7 signal is cut off. Stop the current imaging experiment, correct the z-stacks for the shift,

and begin imaging again. If the embryo shifts too much (i.e., many z-stack changes in a short amount of time), use less immersion oil on the microscope objective lens.

10. If no signal is detected, try modestly increasing the laser power. Otherwise, the His2AV-eBFP2, MCP-GFP, PCP-mCherry flies can be crossed first with wildtype (*OreR* or *yw*) flies to reduce the background levels of MCP and PCP. The resulting virgin progeny can then be crossed to the MS2- or PP7-tagged males. Alternatively, a weak promoter can also be used to drive weaker MCP and PCP expression.
11. Adjusting the color histogram range in Fiji during post-image processing does not change the actual fluorescence intensity signal data of the images. The image files should only be saved as TIFF/TIF for quantification, and not as PNG or JPEG to avoid permanently changing the fluorescence intensity values. In order to store the complete metadata from the experiment, do not change the bit (16 bit) information.

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Figures

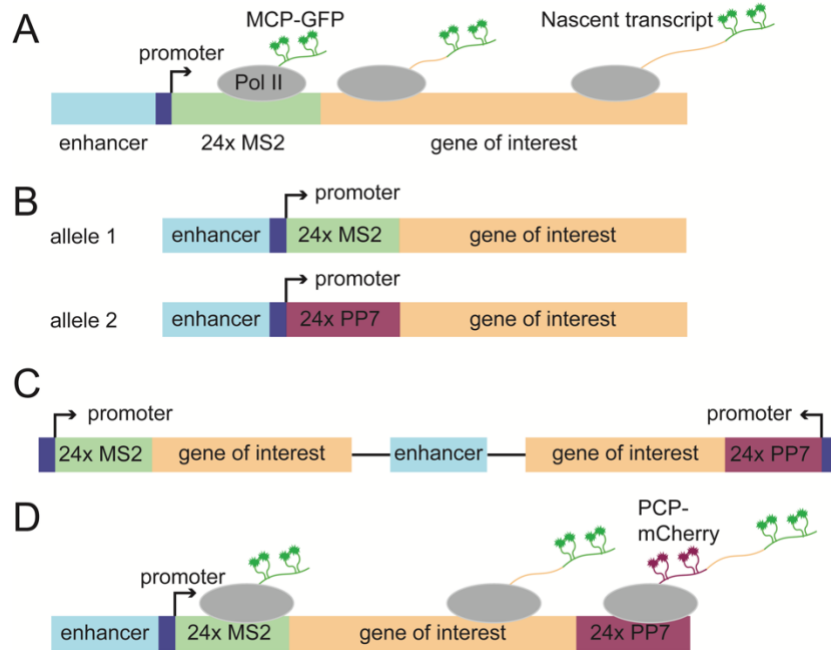


Figure 1. Multi-labeling live imaging using MS2- and PP7-tagged alleles

- (A) Schematic of a representative reporter construct with 24 repeats of MS2 in the 5' UTR of a gene of interest. Upon transcription, MS2 repeats form stem loops, which are bound to by two copies of MCP-GFP. Nascent transcripts are visualized as fluorescent puncta.
- (B) Schematic of a construct to visualize transcriptional activities of multiple alleles in a single nucleus. One allele is tagged with MS2, while the other is marked by PP7.
- (C) Schematic of a construct to visualize transcriptional activities of two promoters driven by a single enhancer. Each promoter-gene module is tagged with either MS2 or PP7.
- (D) Schematic of a reporter construct to measure the rate of Pol II elongation. MS2 and PP7 repeats are inserted into the 5' and 3' UTR of a gene of interest, respectively.

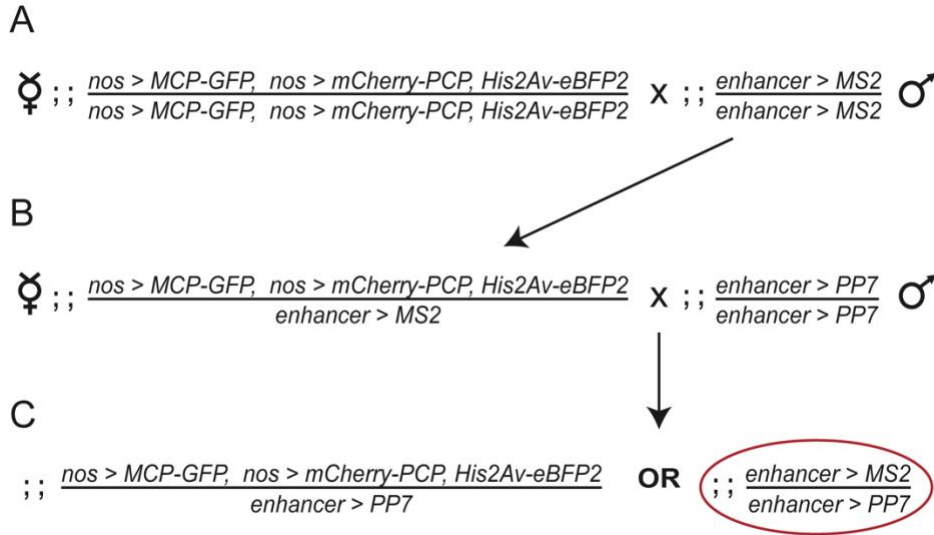


Figure 2. Fly crosses to image allele-specific transcriptional activity

(A) *nos > MCP-GFP, nos > mCherry-PCP, His2Av-eBFP2* homozygous virgins are crossed with homozygous males containing the MS2-tagged gene of interest.

(B) The cage is set up with the virgin progeny from the cross in (A) and homozygous males containing the PP7-tagged gene of interest.

(C) Genotypes of the progeny from the cross in (B) will consist of *nos > mCherry-PCP, His2Av-eBFP2 / enhancer > PP7* that only exhibit PP7 fluorescence, or *enhancer > MS2 / enhancer > PP7* that exhibit both MS2 and PP7 fluorescence.

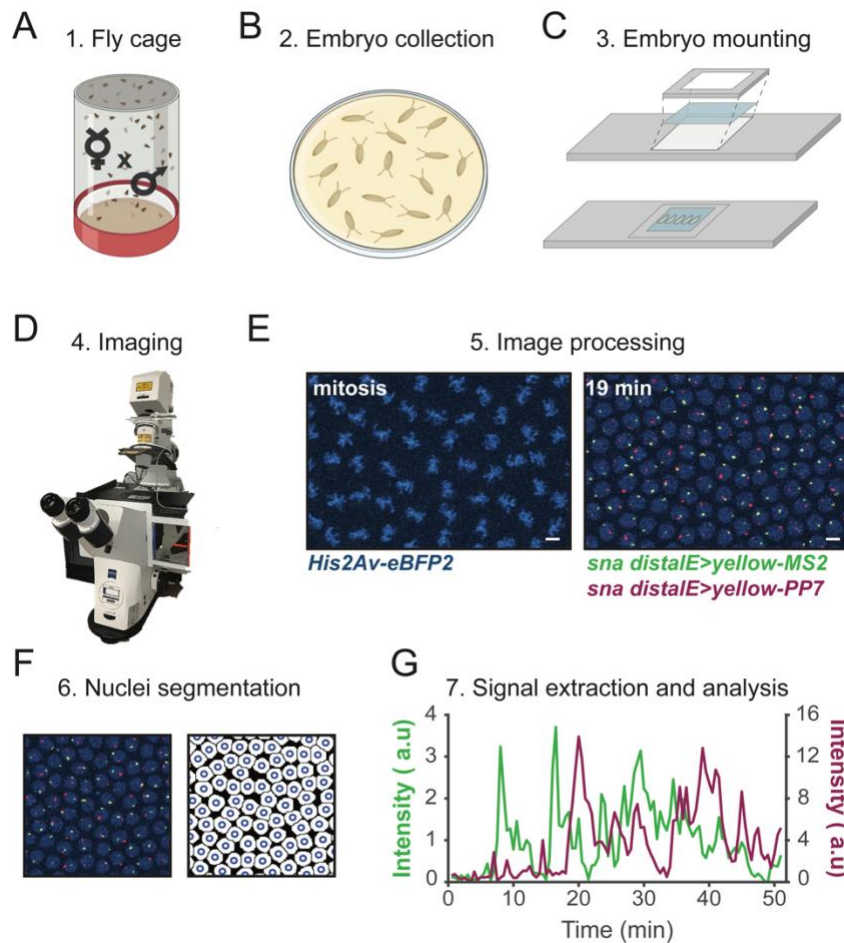


Figure 3. Overview of the live imaging experiment

- (A) The cage is set up with *nos > MCP-GFP*, *nos > mCherry-PCP*, *His2Av-eBFP2 / enhancer>MS2* virgins and *enhancer>PP7 / enhancer>PP7* males.
- (B) Embryos laid during the desired time window are collected and dechorionated.
- (C) Dechorionated embryos are mounted on a membrane that is fitted between a microscope slide and insert.
- (D) Embryos are imaged for the entirety of the developmental time of interest using a confocal microscope.
- (E) The images taken using the microscope are merged to create maximum intensity z-projection images. Nuclei were visualized using His2Av-eBFP2 and are shown in blue. Alleles express the *MS2-yellow* (green) or *PP7-yellow* (magenta) reporter genes driven by the *snail distal* enhancer (*sna distalE*).
- (F) Left, original maximum intensity projection image. Right, image after watershed segmentation and nucleus tracking. The white circle is the mask for a single nucleus. The blue circles indicate that the nucleus was tracked for the entirety of the experiment.
- (G) Representative *MS2-yellow* and *PP7-yellow* transcriptional trajectories from a nucleus shown in (E). The fluorescence signals are extracted and analyzed using MATLAB or other programming software.

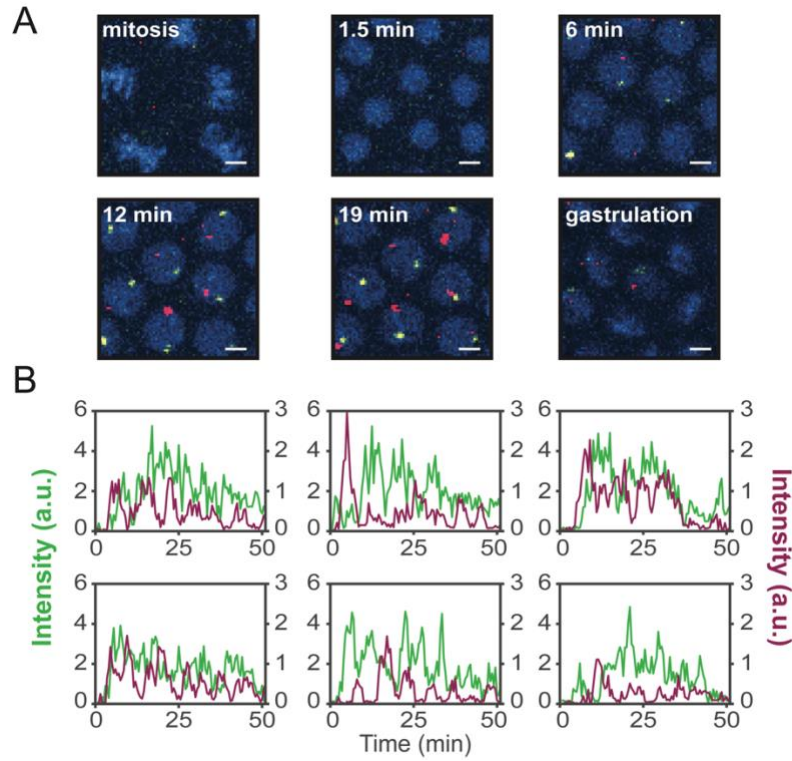


Figure 4. Nuclei exhibit stochasticity throughout transcription

- (A) Snapshots from an embryo expressing *sna distalE>MS2-yellow* and *sna distalE>PP7-yellow* (green and magenta, respectively) at different time points, from the 13th mitosis to gastrulation. Time represents minutes after the 13th mitosis.
- (B) Fluorescence signal trajectories for both *MS2-yellow* (green) and *PP7-yellow* (magenta) for six of the nuclei shown in (A). This kinetic data illustrates the stochasticity of transcriptional activity.

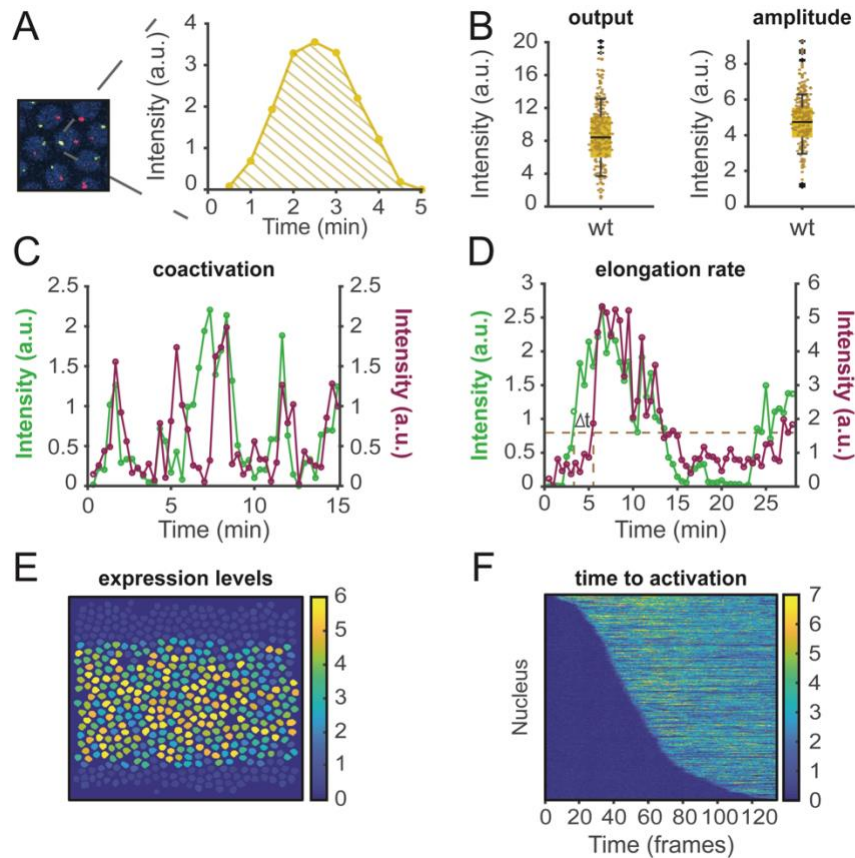


Figure 5. Live imaging provides a platform for analyzing kinetic and spatial information

- (A) Fluorescence signals can be extracted and plotted against developmental time. Since the signal is a proxy for nascent transcription, integrating under the trajectory curve yields a cumulative mRNA output for a single nucleus.
- (B) mRNA outputs from the calculations performed in (B) can be represented in a boxplot. To show the distribution of the data, individual data points can be overlaid on the boxplot. Similarly, the maximum amplitude value of each nucleus can be calculated and plotted.
- (C) Representative nuclear traces of a single enhancer coactivating two promoters simultaneously in a single nucleus. Note regions of coordinate bursting.
- (D) Representative nuclear traces from an embryo expressing a gene tagged with MS2 and PP7 on the 5' UTR and 3' UTR of the gene, respectively. The transcriptional traces can be used to calculate the elongation rate of Pol II by dividing the length of the gene by the time delay between the two traces, Δt .
- (E) Heatmap showing mRNA output of individual nuclei throughout the *snail* expression pattern. Nuclei producing higher levels of mRNA are generally located in the center of the expression domain. The color bar represents the total cumulative output per nucleus (a.u.).
- (F) Heatmap of transcriptional trajectories from each nucleus sorted by the timing of transcriptional activation times (i.e., nucleus 1 is activated earliest). The color bar represents the fluorescence intensity (a.u.).

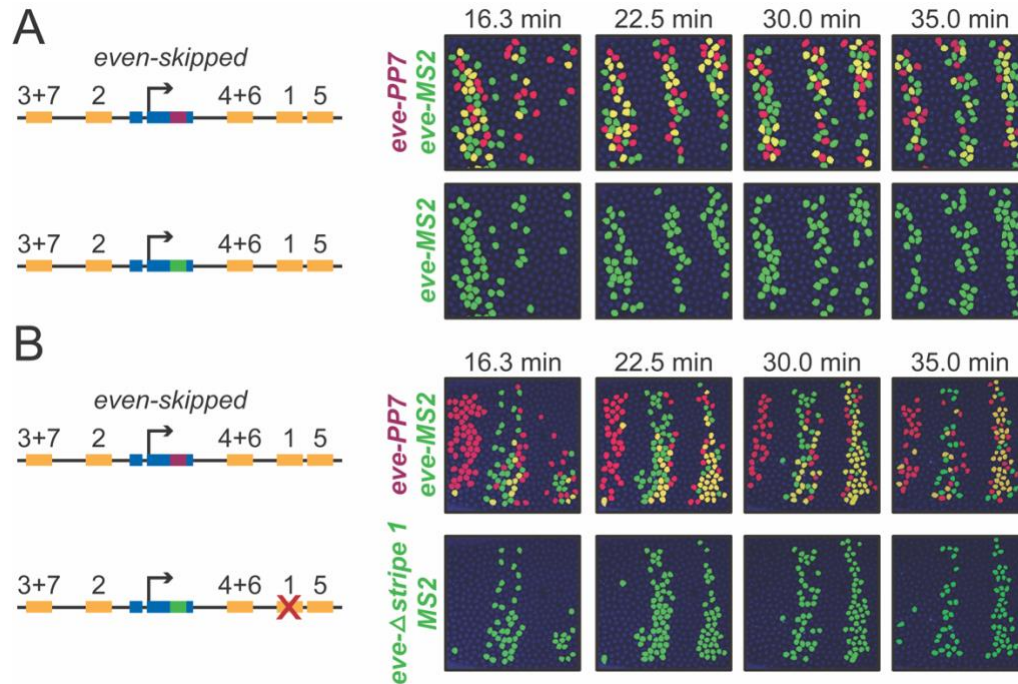


Figure 6. Multi-labeling can be used to visualize wildtype and mutant alleles within a single nucleus

Left, (A) schematic of the *eve* gene and its enhancers and (B) with stripe 1 enhancer removed . The wildtype *eve* is tagged with PP7 on one allele (top) in both (A) and (B). The other wildtype allele (A) or mutant allele (B) is tagged with MS2 (bottom). Right, false-colored snapshots showing both *eve-PP7* (red) and *eve-MS2* (green) activities (top) or only *eve-MS2* activity (bottom).

Note the complete removal of *eve* stripe 1 expression and ectopic expression in the stripe 2 domain. Nuclei that exhibit both PP7 and MS2 signals are shown in yellow. Nuclei were visualized with His2Av-eBFP2 and are shown in blue. Time indicates minutes after onset of nuclear cycle 14. Images were rotated to orient the embryo (left-anterior, right-posterior).