Live Imaging of mRNA Synthesis in *Drosophila*

Hernan G. Garcia and Thomas Gregor

Abstract

mRNA synthesis is one of the earliest readouts of the activity of a transcribed gene, which is of particular interest in the context of metazoan cell fate specification. These processes are intrinsically dynamic and stochastic, which makes in vivo single-cell measurements inevitable. Here, we present the application of a technology that has been widely used in single celled organisms to measure transcriptional activity in developing embryos of the fruit fly *Drosophila melanogaster*. The method allows for quantification of instantaneous polymerase occupancy of active gene loci and thereby enables the development and testing of models of gene regulation in development.

Key words Nascent mRNA, Transcription activity, Quantitative live imaging, Fluorescence, *Drosophila*

1 Introduction

The observation of mRNA transcript production in real time has become a familiar routine in the context of single cells [1–3]. This method entails the use of an mRNA tagging system in which nascent transcripts are tagged with multiple repeats of a stem loop sequence that is recognized by a cognate binding protein (Fig. 1a). The latter is constitutively expressed and fused to a fluorescent protein that can be visualized using standard live microscopy techniques [4–6]. The stem loop cluster binds multiple fluorescent proteins resulting in spatially localized fluorescence at the gene locus, which is further enhanced by each additional polymerase that is engaged in transcriptional elongation (Fig. 1b). In the fruit fly *Drosophila melanogaster*, this MS2 system has been implemented to study maternal mRNA transport in oocytes [7] and transcription [8–13].

Here we present a detailed protocol to visualize and quantify nascent transcripts in living fly embryos using the MS2 stem loop system (Fig. 1c). Details are provided regarding the transgenic
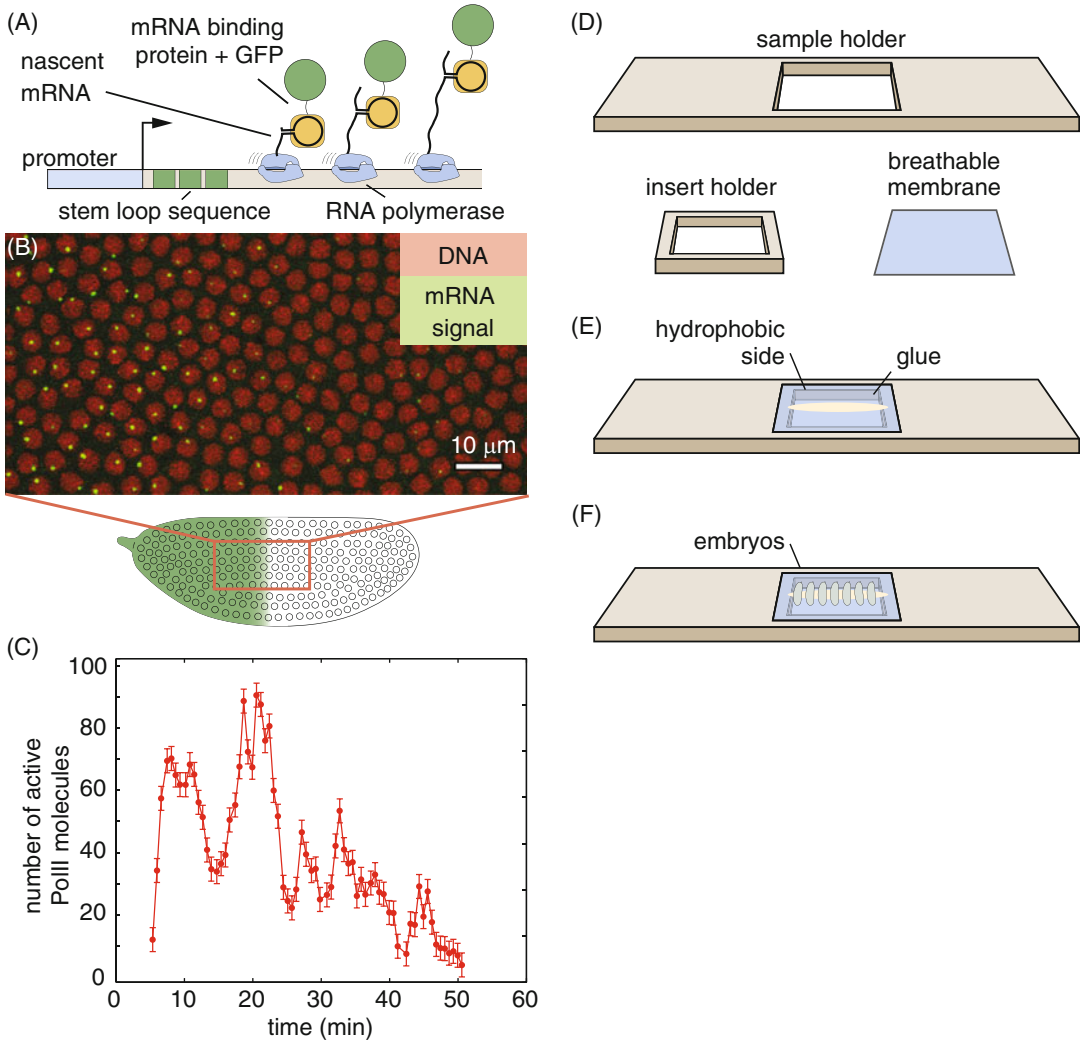
Fig. 1 Quantifying transcriptional dynamics in live fly embryos. (**a**) 24 repeats of the MS2 loop sequence are added to a gene that, when transcribed, fold into a stem loop that is recognized by an mRNA binding protein fused to GFP; fluorescence is proportional to transcriptional activity. (**b**) Typical field of view showing sites of transcription in single nuclei. (**c**) Number of actively transcribing Pol II molecules as a function of time for a labeled site of nascent transcript formation. (**d–f**) Sample holder and breathable membrane for embryo mounting

construct design and implementation, embryo handling for live imaging, and various suitable microscopy techniques. We discuss confocal and two-photon imaging conditions for optimal high quality images for quantitative analysis. In particular, we discuss calibration of the fluorescence signal to obtain absolute units in terms of numbers of actively transcribing RNA Polymerase II (PolII) molecules.

2 Materials

2.1 Creating MS2 Reporter Constructs and Transgenic Fly Lines

1. Plasmid pCR4-24XMS2SL-stable [4] (Addgene #31865) to make a new MS2 reporter construct.

2. Plasmids pIB-hbP2-P2P-lacZ-αTUb3′UTR [14] or pBφ-eve2-MS2-yellow [10] for plasmids ready for transgenesis using recombination mediated cassette exchange (RMCE) [15] or single attP site integration [16], respectively.

3. Transformation competent *E. coli* (e.g., XL1-Blue).

4. Stbl2 competent *E. coli* cells (Life Technologies 10268-019).

5. Bloomington *Drosophila* stock center fly lines #27388 (for RMCE) or #9750 (single attP).

6. In-house microinjection setup to generate transgenics or third-party company such as Bestgene (www.thebestgene.com) or Rainbow Transgenics (www.rainbowgene.com).

2.2 Fly Lines

1. yw;His2Av-mRFP;Pnos-MCP-EGFP [8] from Bloomington *Drosophila* Stock Center (#60340) expresses MCP-GFP and Histone-RFP maternally.

2. yw;P2P-MS2-lacZ and yw;P2P-lacZ reporter fly lines [8] for absolute calibration of PolII molecules actively transcribing (available upon request).

2.3 Embryo Collection

1. Apple juice agar plates.

2. Active dry yeast (e.g., Fleischmann's ADY 2192).

3. Halocarbon oil 27 (e.g., Sigma-Aldrich).

4. Absorbent paper towels.

5. 20% bleach.

6. Distilled or reverse osmosis water.

2.4 Embryo Mounting

1. Breathable Lumox Film (e.g., Sarstedt 94.6077.317).

2. Double-sided sticky tape.

3. 50 mL conical tubes.

4. 1.5 mL Eppendorf tubes.

5. Heptane.

6. Platform rocker (Nutator).

7. Tabletop centrifuge.

8. Scintillation vial.

9. Embryo and membrane slide holder (http://www.sculpteo.com/en/gallery/public/hggarcia/).

10. Dumont #5 forceps.

11. Round brush (size 0 or smaller).

2.5 Imaging	1. Custom-built 2-photon microscope [17] with a Zeiss 25×0.8 NA objective, GaAsP photomultipliers (Hamamatsu H10770PA-40 SEL), Pockel Cell or neutral density filters to modulate laser output, Coherent power meter #1159770 and ScanImage control software (www.scanimage.org).
	2. Confocal microscope with high sensitivity photodetectors. We use a Leica SP8 with $63 \times$, 1.4 NA objective, Argon and Helium-Neon lasers, or white light laser and HyD detectors and a Zeiss 780 with $40 \times$, 1.4 NA objective, Argon and Helium-Neon lasers and HyD detectors.
	3. Diagnostic slides for measuring flat field (Chroma #92001).
2.6 Data Analysis	1. Mathworks Matlab.
	2. FlyRNAQuant(github.com/PrincetonUniversity/FlyRNAQuant).

3   Methods

The protocol begins with the creation of transgenic *Drosophila* lines bearing stem loop-tagged reporter constructs. The mounting of embryos onto a custom-made slide sample holder (Fig. 1d–f) is described. This sample holder makes it possible to flatten the embryos and have as many nuclei as possible in the same focal plane. Lastly, the imaging protocol for the measurement of transcriptional activity in live embryos is described.

3.1 Creating DNA Reporter Molecules	1. Use the unique restriction sites in pIB-hbP2-P2P-lacZ-α TUb3′UTR (RMCE) or pBφ-eve2-MS2-yellow (single attP insertion) to insert new regulatory regions using regular cutting-and-pasting with restriction enzymes or using Gibson assembly (vector maps available at benchling.com/garcialab).
	2. Transform the newly generated plasmids into supercompetent cells such as XL1-Blue.
	3. Some of the MS2 stem loops can be lost during the previous step. Before sequencing colonies screen them by digesting candidate plasmids using restriction enzymes such as EcoRV and running an analytical gel. Send for sequencing with primers for the new insert and for the stem loops.
	4. Once sequencing is confirmed transform the plasmid into Stbl2 cells for archival purposes.
	5. Generate transgenic flies by injecting the newly generated vector in-house or through a company.
3.2 Embryo Glue	1. Densely pack a 50 mL conical tube with strips of double-sided sticky tape.
	2. Fill the tube with heptane.

3. Mix the tube overnight on a nutator.

4. Using forceps extract the tape and pipette the heptane into Eppendorf tubes.

5. Spin down the tubes at maximum speed on a tabletop centrifuge.

6. Pipette the supernatant into a scintillation vial and store at 4 °C.

3.3 Preparing the Sample Holder for Live Imaging of Embryos

1. Identify the hydrophobic side of the permeable membrane using a sharpie: it will be harder to write on the hydrophobic side.

2. Cut a 2 cm × 2 cm square.

3. Mount the membrane into the membrane holder as shown in Figs. 1d and e.

4. Apply a thin line of glue to the membrane (Fig. 1e). This glue will avoid embryo rolling during sample preparation.

3.4 Preparing Transgenic Embryos for Live Imaging

1. Two days before imaging prepare a cage of flies containing 50–100 virgin females of His-RFP;MCP-GFP flies and 20–40 males containing the reporter construct.

2. 90 min before preparing the embryos replace the plate in the cage for a new one with fresh yeast.

3. After the waiting time replace the plate again.

4. Cover plate with halocarbon oil and image embryos using a dissecting scope with trans-illumination (*see* **Note 1**).

5. Using the forceps pick early embryos [18] and transfer them to a small (1 cm × 1 cm) cutout of a paper towel.

6. Cover with bleach for 1 min.

7. Absorb bleach using a paper towel and wash with water for 1 min.

8. Absorb water and transfer paper towel to a petri dish with water. Undamaged embryos will float.

9. Using the brush transfer embryos to a small dry cutout of paper towel.

10. Pick embryos one by one in the right orientation using the brush and place them on the glue on the membrane (Fig. 1f) (*see* **Note 2**).

11. Put two drops of halocarbon oil on embryos and place an 18 × 18 mm 1.5 cover glass on them. Let the sample sit for 1 min.

12. Inspect the samples for embryo flattening. If embryos need further flattening use the side of a Kimwipes to absorb oil from the side of the cover glass. Monitor cover glass so that it does not slide and the embryos so that they do not explode.

13. This sample can be used both on an upright or inverted microscope configuration.

3.5 Setting Up the Microscopes for Imaging

Turn on the microscope system and lasers at least 90 min before imaging the embryos.

3.5.1 2-Photon Microscopy for Live Imaging

1. Set the TiSa laser wavelength to 970 nm.
2. Set up power meter at the objective exit.
3. Park scanner or set it to a high zoom of 80 or larger and scan.
4. Measure power and use the Pockel Cell or the neutral density filter to adjust it to 10 mW.
5. Set scanning to a pixel size of 220 nm, 512 pixels per line, 256 lines per frame, and a frequency of 250 Hz per line (4 ms/line).
6. Set Z-stack continuous acquisition to ten slices separated by 1 µm, each slice is averaged three times.

3.5.2 Confocal Microscopy for Live Imaging

1. Use a 488 nm and a 561 nm laser excitation lines.
2. Set up power meter at the 10× objective exit, zoom to 40×, and find the right laser power settings for a 35 µW output power in 488 nm and 20 µW output power in 561 nm.
3. Set scanning to a pixel size of 220 nm, 512 pixels per line, 256 lines per frame and a frequency of 400 Hz per line and bidirectional scanning.
4. Set Z-stack continuous acquisition to 21 slices separated by 0.5 µm, each slice is averaged three times.

3.6 Live Imaging

1. Find the embryos using brightfield illumination and save their positions using an automated XY stage.
2. Using fluorescence illumination look for an embryo whose nuclei are just migrating to the surface.
3. Find the nuclei and transcription spots, and start a continuous acquisition by centering the Z-stack on the middle of the nuclei (*see* **Notes 3** and **4**).
4. The sample should be monitored regularly as nuclei and their transcription spots tend to drift during acquisition. Adjust the center of the Z-stack as needed throughout the experiment.
5. Once done with the acquisition take an image of the full embryo image in order to determine the correct AP position of the zoomed-in image and to check for photobleaching (*see* **Note 5**).
6. Using the same acquisition parameters of the Z-stack measure the flat field using diagnostic slides for measuring flat field (*see* **Note 6**).

3.7 Live Data Analysis	1. Download the FlyRNAQuant software and manual.
	2. Install the software as instructed in the manual and download a sample data set.
	3. Follow the analysis of the sample data set all the way to the end to ensure the full functionality of the code.
	4. Transfer the obtained data to the analysis computer with analysis software.
	5. Go through the different steps detailed in the manual for the automated analysis.
	6. If single cell information is required, particular attention needs to be paid to the curation step of the traces.
3.8 Calibration of Absolute Number of Active PolII Molecules	1. Measure the transcriptional activity of reporter fly line P2P-MS2-lacZ as described in Subheadings 3.3, 3.4, 3.6, and 3.7.
	2. Integrate the fluorescence over nuclear cycle 13 to infer the number of total number of mRNA molecules produced as described in [8].
	3. Measure the number of mRNA molecules produced by the reporter line P2P-lacZ in the cytoplasm during mitosis 12 and mitosis 13 using the single-molecule FISH (smFISH) protocol described in Chapter 8 of this book [19].
	4. Take the difference of mRNA molecules obtained by smFISH during mitosis 12 and 13 and compare it to the quantity obtained by live imaging during the same developmental stage in order to calibrate the arbitrary fluorescence units of the MS2-MCP-EGFP reporter [8].

4   Notes

1. The halocarbon oil allows to easily image through the embryos and score their developmental time.

2. Mounting a typical amount of 16 embryos usually ensures finding several in the right orientation and developmental stage for imaging.

3. If no spots or low fluorescent signal is detected, check and/or increase laser power. Image the flat field slide as a means to make sure that the detectors are working properly.

4. If the imaging conditions are checked and the problem of no/low signal persists, it is possible that the MS2 stem loops were lost during the cloning procedure. Check the number of loops as in **step 3** of Subheading 3.1 both in the transgenic fly line, and the plasmid originally used to generate this line. If necessary, rescreen bacterial colonies.

5. If pronounced photobleaching on the MCP-GFP channel is detected reduce the laser power and/or reduce the frequency of acquisition.

6. The laser power will have to be reduced dramatically so as to not saturate and damage the detectors.

Acknowledgments

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