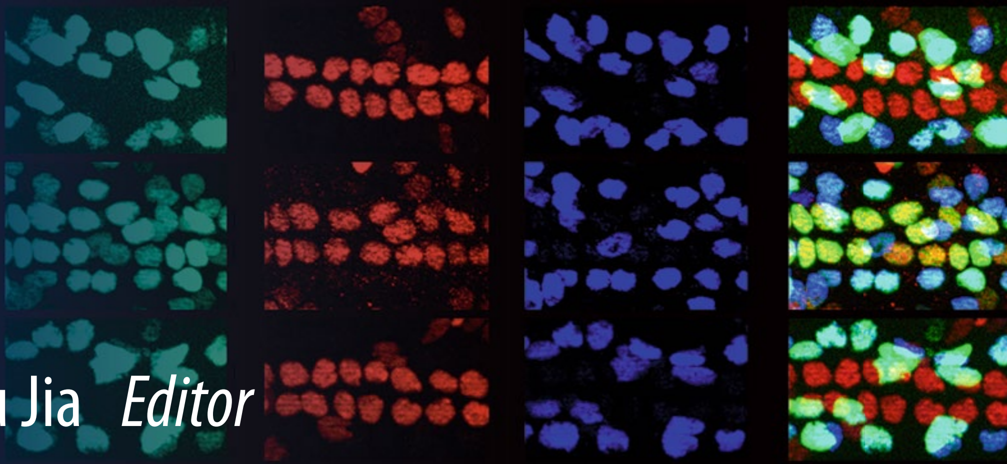
Methods in
Molecular Biology 2472

Springer Protocols



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Chapter 11

Image-Based Single-Molecule Analysis of Notch-Dependent Transcription in Its Natural Context

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Abstract

Notch signaling is crucial to animal development and homeostasis. Notch triggers the transcription of its target genes, which produce diverse outcomes depending on context. The high resolution and spatially precise assessment of Notch-dependent transcription is essential for understanding how Notch operates normally in its native context in vivo and how Notch defects lead to pathogenesis. Here we present biological and computational methods to assess Notch-dependent transcriptional activation in stem cells within their niche, focusing on germline stem cells in the nematode *Caenorhabditis elegans*. Specifically, we describe visualization of single RNAs in fixed gonads using single-molecule RNA fluorescence in situ hybridization (smFISH), live imaging of transcriptional bursting in the intact organism using the MS2 system, and custom-made MATLAB codes, implementing new image processing algorithms to capture the spatiotemporal patterns of Notch-dependent transcriptional activation. These methods allow a powerful analysis of in vivo transcriptional activation and its dynamics in a whole tissue. Our methods can be adapted to essentially any tissue or cell type for any transcript.

Key words smFISH, MS2, Confocal, Wide-field microscopy, Germline stem cells (GSC) single-molecule visualization, Transcriptional dynamics, Transcriptional bursting, RNA live imaging, Whole worm live imaging

1 Introduction

Notch signaling is a highly conserved cell–cell signaling pathway that is crucial to metazoan development and homeostasis [1–3]. Dysfunction or misregulation of Notch can lead to a variety of human pathologies, including cancer, congenital heart defects, and neurodegenerative diseases [4–10]. Notch signaling works by activating transcription of its target genes, whose products regulate key biological processes such as stem cell maintenance and tissue repair [11–14]. Physical interaction between a Notch ligand on a signaling cell and a Notch receptor on a receiving cell triggers cleavage of the receptor, allowing the Notch intracellular domain (NICD) to enter the nucleus and form a ternary complex with the

Dongyu Jia (ed.), *Notch Signaling Research: Methods and Protocols*,
Methods in Molecular Biology, vol. 2472, https://doi.org/10.1007/978-1-0716-2201-8_11,

DNA-binding protein, CSL/RBPJκ and the coactivator, MAML [2]. That complex then activates transcription of its target genes [15]. Therefore, Notch target transcripts are a hallmark of Notch activation. The transcriptional response to Notch signaling has been analyzed in diverse organisms, including *Drosophila* and zebrafish [16–20], and our recent work in *C. elegans* germ cells highlights the power of single-molecule analyses done at the resolution of nascent transcripts at individual Notch target loci in their native context [21–24]. We used single-molecule Fluorescence In Situ Hybridization (smFISH) to visualize both nascent transcripts in the nucleus and mature mRNAs in the cytoplasm at the single-RNA molecule resolution in an intact tissue [25–28]. Importantly, this method detects transcripts from the endogenous wild-type gene rather than relying on an artificial or indirect reporter, an approach that monitors the Notch transcriptional response in its authentic in vivo setting. In addition, we developed a computational method with custom-made image processing algorithms to automate the detection of RNA molecules in the whole tissue with high precision for their multidimensional spatial pattern analysis [21]. We developed these biological and computational methods to detect and analyze the Notch transcriptional response in the *C. elegans* germline, where Notch signaling is required to maintain germline stem cells (GSCs) [11, 29]. Recently, we successfully adapted our approach to other tissues in *C. elegans* (e.g., intestine and seam cells, unpublished) and to human and mouse cells in culture (e.g., induced pluripotent stem cells and cardiomyocytes [30]) with a few modifications and optimization steps in both biological and computational methods.

Our previous work with smFISH for the Notch target *sygl-1* revealed the probabilistic and stochastic nature of Notch-dependent transcription in GSCs [21–24]. The Notch-dependent transcriptional response is graded within the GSC pool, with its probability higher near the stem cell niche. However, not all GSCs, even GSCs adjacent to the niche, are transcriptionally active. Furthermore, the transcriptional activity (number of *sygl-1* nascent transcripts generated) at each locus is highly variable. There was no clear pattern or correlation with other critical factors such as cell cycle, cell size, or location to explain the variability in *sygl-1* transcription [21].

These results led us to the idea that the Notch-dependent transcription may occur in bursts. To test this idea, we developed a whole animal live imaging system in *C. elegans* using the MS2 system coupled to a worm immobilization method [24]. The MS2 system takes advantage of the strong and specific binding of the MS2 Coat Protein (MCP) to an array of tandem repeats of MS2 loops that are inserted into the target locus [31, 32]. We introduced 24 MS2 loops into a Notch target gene, *sygl-1,* as a transgene to visualize its transcripts in the nucleus. Combining the MS2 system, intact worm live imaging, and our computational method, we could trace *sygl-1* nascent transcripts for over 10 h. We thus were able to analyze the dynamics of Notch-dependent transcription at each locus in its natural setting. Notch indeed activates transcription in a bursty manner. Moreover, the strength of Notch modulates the transcriptional burst duration rather than the frequency, which is unexpected from previous in vitro studies about regulation of transcriptional dynamics [24, 33–35].

Here, we present our methods of single-RNA molecule visualization in fixed tissues (Subheading 3.1) and long-term whole animal live imaging using the MS2 system (Subheading 3.2). For the smFISH method, we include updates and greater depth than our previously published method [23]. We also present our computational methods to detect Notch-dependent transcription (Subheadings 3.1 and 3.2). The information of detected transcripts and nuclei such as their 3D coordinates and signal intensities can be used for further analyses as in our previous publications [21–23, 26, 30]. These methods can be used in any tissue or cell type for any transcripts after optimization.

2 Materials

2.1 smFISH in the C. elegans *Germline*

1. RNase-free microcentrifuge tubes (1.5 mL) (*see* **Note 1**).
2. Microscope slides.
3. Microscope cover glass with refractive index matching immersion oil for the microscope in use.
4. Aluminum foil.
5. Scalpel (Feather disposable scalpel #10).
6. RNase-free filtered tips (20, 200, and 1000 μL tips).
7. smFISH probe(s) conjugated with fluorophores (LGC BioSearch Technologies, https://www.biosearchtech.com/),
8. RNaseZap®.
9. ProLong Gold Antifade Reagent Mountant.
10. Nail polish (transparent).
11. Levamisole (or Tetramisole).
12. 37% formaldehyde
13. Ethanol (200 proof).
14. 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI)
15. TE buffer (pH 8.0, RNase-free): 10 mM Tris base, 1 mM EDTA.
16. PBST: RNase-free 1× phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4) + 0.1% Tween 20.

17. Permeabilization buffer: RNase-free 1× PBS + 0.1% Triton X-100.
18. Hybridization buffer (10 mL, RNase-free): 1 g dextran sulfate, 10 mg *E. coli* tRNA, 100 μL vanadyl ribonucleoside complex (200 mM), 40 μL nuclease-free BSA (50 mg/mL), 1 mL 20× SSC1 mL deionized formamide, 7.3 mL RNase-free H_2O (up to 10 mL volume). Bring all reagents to room temperature before making the hybridization buffer (*see* **Note 2**).
19. Wash buffer (RNase-free): 1 mL RNase-free 20× SSC, 1 mL deionized formamide, 8 mL RNase-free H_2O, 10 μL Tween 20. Bring all reagents to room temperature before making the wash buffer.

2.2 MS2 RNA Live Imaging

1. VALAP: Vaseline, lanolin, paraffin (1:1:1 w/w/w), melt together on a heat block at a low to medium setting (60–80 °C) and store at room temperature [24].
2. Polystyrene microbead (0.1 μm diameter), stored at 4 °C.
3. M9 buffer (3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 ml 1 M $MgSO_4$, H_2O to 1 L). Sterilize by autoclaving.
4. 5% agarose.
5. Microscope slide.
6. Microscope cover glass.
7. Serotonin creatinine sulfate monohydrate. A small quantity of freshly made 50 mM serotonin solution (dissolved in M9) is needed for each experiment.
8. Optional: CherryTemp (microscope slide temperature controller, Cherry Biotech). Any temperature controller can be used.

2.3 MATLAB Codes and ImageJ Macros

1. All MATLAB codes and ImageJ macros needed for smFISH or MS2 data analysis can be found at: https://github.com/chlasic
2. smFISH_detection_analysis package (https://github.com/chlasic/smFISH_detection_analysis),
3. MS2_analysis package (https://github.com/chlasic/MS2_analysis).

3 Methods

3.1 smFISH in the *C. elegans* Germline

Samples are centrifuged at 2000 rpm for 30–60 s, unless otherwise stated.

3.1.1 smFISH Procedure

Probe Stock

1. Dissolve the dried probe mix (5 nmol) in 40 μL of RNase-free TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) to create a master probe stock of 125 μM (*see* **Note 3**).

2. Dilute the master stock 1:20 in RNAse-free TE buffer as a working stock. For the experiment, 1 μL of working stock is added to 49 μL of sample (1:50) for a final probe dilution of 1: 1000 and a final probe concentration of 125 nM (*see* **Note 4**).

Preparation for smFISH

1. Wipe down bench top, gloves, and pipettors with RNaseZap@ Wipes or spray. Rewipe gloves and pipettors if needed or exposed to RNases. Use RNase-free filtered tips and RNase-free tubes (*see* **Note 1**) and keep tip boxes closed when not in active use.
2. Prepare PBST (RNase-free 1× PBS + 0.1% Tween-20), permeabilization buffer (RNase-free 1× PBS + 0.1% Triton X-100), and RNase-free 70% ethanol, and 37% formaldehyde beforehand. These reagents can be premade and stored at 20 °C for at least a year.

Dissection of *C. elegans* Gonads

1. Prepare plate with 50–100 staged *C. elegans*. Collect worms by washing with PBST (can be nonsterile) using a P-1000 pipette and transfer the worm suspension to a 60-mm plastic petri dish cover or 100-mm glass petri dish (*see* **Note 5**). We typically use 4 mL worm suspension containing 50–100 worms in a in a 60-mm dish cover.
2. Add 0.25 mM levamisole (1:1000 from 0.25 M stock) directly to the worm suspension. For example, add 4 μL levamisole stock to a 4 mL worm suspension.
3. Using a scalpel, dissect worms by cutting behind the pharynx or before the rectum to release gonads. After levamisole treatment all dissection needs to be done within 20 min to prevent gonad deformation (*see* **Note 6**).
4. Collect dissected worms with pipette and transfer them to a new RNase-free 1.5 mL microcentrifuge tube or a new 15 mL conical tube (*see* **Note 1**). Avoid pipetting up intact (undissected) worms unless you plan to use them for your experiment.
5. Spin worm solution for 15 s at 2000 rpm. Remove supernatant and resuspend with 1 mL PBST.

Fixation and Permeabilization

1. Add formaldehyde (final concentration 3.7%. c.f., 1:10 dilution from 37% formaldehyde stock) directly to the dissected worms in PBST. For example, add 100 μL 37% formaldehyde to a 1 mL sample.
2. Incubate for 15–45 min at room temperature. We typically use 30 min. Rotate tubes (low rpm) or gently invert a few times every ~10 min.
3. Spin worm solution. Remove formaldehyde and dispose in the organic waste bottle. After this step, all reagents must be

RNase-free except for microscope slides and cover glass (*see* **Note 1**).

4. Add 1 mL permeabilization buffer (RNase-free 1× PBS + 0.1% Triton X-100) to the sample and incubate for 10 min at room temperature to permeabilize.
5. Wash with 1 mL PBST. Invert 5–6 times and spin at 2000 rpm for 30–60 s at room temperature. Repeat once for a total of two washes.
6. Resuspend the sample in 1 mL 70% EtOH and incubate at 4 °C for more than 4 h and less than 1 week.

smFISH Probe Hybridization (Day 2)

1. Bring hybridization buffer (HB) to room temperature before opening (*see* **Notes 2, 7,** and **8**). Thaw working probe stock, protecting from light. Probe dilutions vary depending on the probe set and need to be optimized before experiment (*see* **Notes 3** and **4**).
2. Spin fixed sample and remove ethanol.
3. Add 1 mL smFISH wash buffer, equilibrate 5 min at room temperature either in a tube rack or with gentle mixing.
4. While samples are equilibrating in wash buffer, mix HB and probe in a separate tube—prepare a new RNase-free 1.5 mL microcentrifuge tube (*see* **Note 1**) and add 49 μL HB + 1 μL probe dilution (*see* **Note 4**). If you multiplex probe sets, add 1 μL of each probe dilution (e.g., 1 μL of probe set 1 and 1 μL of probe set 2 in 48 μL of HB). If you have multiple samples (multiple tubes) prepare more HB solution (e.g., 392 μL HB + 8 μL probe dilution for eight samples). Vortex the solution and snap the tube to bring all solution to the bottom of the tube.
5. Spin down the fixed dissected worms and remove the wash buffer.
6. Add 50 μL of HB/probe solution in the tube containing sample. Gently flick the tube 5–6 times. Avoid making bubbles.
7. Wrap the tube with aluminum foil or place it in a sealed box to protect from light and incubate for more than 4 h to overnight at 37 °C on rotator (*see* **Note 9**).

Sample Washing and Mounting (Day 3)

1. All incubations should be done in the dark (*see* **Note 8**).
2. Add 1 mL wash buffer to sample, invert 5–6 times to mix. Spin and remove buffer.
3. Add 1 mL wash buffer to sample and incubate for 30 min on rotator at room temperature.
4. Spin down and remove the wash buffer.

5. Add 1 mL wash buffer +1 μg/mL DAPI and incubate for 30 min on rotator at room temperature. (*see* **Note 10**).
6. Wash 2× with 1 mL wash buffer (invert tube 5–10 times for each wash).
7. Mount samples on a microscope slide using ProLong Gold (PLG) Antifade Mountant (Life Technologies) (*see* **Note 11**).
8. Cover with 18 × 18 or 22 × 22 mm square cover glass. We use 10 μL or 12 μL PLG for the respective sizes (*see* **Note 12**).
9. Place the microscope slide on a flat surface in the dark, for example in a drawer at room temperature for 10–20 min, then gently push down the coverslip using a pipette tip to push out big air bubbles if present.
10. To cure the sample, leave the microscope slide in a dark place (in a box or a drawer) for at least 24 h (*see* **Note 13**).
11. Remove excess PLG on the slide by gently pushing down the edges of cover glass if needed. Seal the sample by putting nail polish around the edge of coverslip. Do not apply excess nail polish but make sure sample is sealed in all sides.
12. The slide can be stored in the dark at room temperature if it will be imaged within a week. Keep slides at 4 °C or, ideally, −20 °C for long-term storage (*see* **Note 14**).

3.1.2 smFISH Imaging

We have successfully imaged smFISH signals both with confocal and wide-field (compound) microscopes [21, 27]. Images taken by wide-field microscopy may need further image processing such as deconvolution. The deconvolution parameters (e.g., iterations and point spread function) must be carefully chosen to avoid over- or underimage processing which can create artificial signals (dots, granules, or tubules). Here, we report the image acquisition settings for both confocal and wide-field microscope systems.

Confocal Microscopy Settings

1. Microscope: Leica SP8 Confocal Laser Scanning microscope.
2. Objective lens: Leica HC PL APO CS2 63×/1.40 OIL.
3. Photon detector: Sensitive hybrid detector (HyD) for smFISH and photomultiplier (PMT) for DAPI.
4. Frequency: 400 Hz.
5. Zoom factor: 3×.
6. Pinhole: typically 95.5 μm (1 airy unit).
7. Laser power: between 1 and 5%. The power changes depending on the fluorophores conjugated to smFISH probes and the light source. For example, the *sygl-1* exon probe (CAL Fluor Red 610) was excited at 594 nm using 1.2% HeNe. Choose settings below saturation (255 for 8-bit images) so that the images can be quantitated.

8. Signal acquisition bandwidth: typically 100 nm. Start the acquisition window 5–10 nm from the end of the excitation laser range. For instance, the *sygl-1* exon probe conjugated to CAL flour 610 was excited with the 594 nm laser and the emission was acquired at 602–702 nm (*see* **Note 3**).
9. Gain: 40.
10. Acquisition settings: line average: 6–8 with 1–2 frame accumulation and unidirectional scanning.
11. *Z* step size: 0.3 μm.

Wide-Field Microscopy Settings

1. Microscope: Leica THUNDER Imager 3D Assay with RFID and LED8 (DMi8).
2. Objective lens: Leica HC PL APO 63×/1.40–0.60 OIL.
3. Filter set: DFT51011 quad cube.
4. LED Light intensity: 35–50%, typically 40%.
5. Exposure time: 150–300 ms, typically 250 ms.
6. *Z* step size: 0.3 μm.

3.1.3 smFISH Image Analysis

The original MATLAB code to process smFISH images and analyze the spatial patterns of RNA spots was presented in our previous work [21]. Here, we describe the latest version of the code (smFISH_detection_v2_7.m). We improved existing spot detection algorithms and implemented a few more tests to ensure the code detects transcripts and nuclei more accurately. It supports Leica image libraries (lif files), as in the previous versions, and has been updated to also handle TIFF format image files (*see* **Note 15**). The code consists of one main script (smFISH_detection.m) and several function codes in a folder called "Function."

1. Download all files and use the main script to run the code for smFISH image analysis on MATLAB. The code is available at: https://github.com/chlasic/smFISH_detection_analysis. New code will be posted when there are new updates.
2. User can choose one of two methods for selecting a folder containing image files with the extension "lif" or "tif" to be processed and another folder for saving the image processing results by using the variable "Method_of_choice" in the line 15 of the main script. Enter "1" if you want to use the user interface system, which will pop up a window for navigating to and selecting the folders. Enter "2" if you want to manually input the folder paths in lines 47 and 60 for the input and output folders, respectively.
3. Set the thresholds for detecting RNAs and nuclei (*see* **Notes 16** and **17**).

(a) Threshold for mRNA detection using exon probes: "thresForExon" (line 20).

(b) Threshold for ATS detection using intron probes: "thresForIntron" (line 21).

(c) Threshold for nuclear detection: "thresForNuc" (line 22).

The default is 1 and user can change the threshold from 0 to infinity. We found that most ~~of~~ detection works with thresholds between 0.3 and 1.8. The user must gauge the thresholds for the best detection results. The function "VisualRNAdetect" can be used to check the detection results. An example usage is "VisualRNAdetect(af, 1, 'ro', 50);". The user can change the second input argument "1" for indicating the *n*th RNA channel to visualize. The fourth argument ("50") is the brightness of the maximum projected image. Threshold levels for each channel are set using the same strategy. We try several thresholds for each channel for 5–10 images and choose a threshold that works best for most of the images within an experimental set. Noninteger values such as 1.25 can be used for the thresholds.

4. Indicate the type of channels in the image such as the exon or intron channel. The variable "ChOrder" in the line 29 of the main script records the channels the image contains. Use "1" for indicating the nucleus channel, "2" for the exon channel, and "3" for the intron channel. Use "5" if you want to skip processing the channel. For example, if you have three channels in your images in the order of (1) a nucleus channel, (2) an exon channel, and (3) an intron channel, use "ChOrder = [1 2 3]; ".

5. Set other parameters for accurate nucleus detection and mRNA counting (the number of mRNA per cell). The preset values work well with *C. elegans* germ cells in the distal gonad. The names of the variables storing these parameters, which are in lines 32–35 in the main script, are listed below. The variables "nrange" and "sensi" are for nucleus detection and "radius" and "vLim" are for mRNA counting in a cell. These parameters must be optimized for smFISH in systems other than the *C. elegans* germline (*see* **Note 18**).

(a) nrange: defines the range of nuclear sizes in μm. The preset is [2.5 4.0] which means nuclei whose diameter is outside the range 2.5–4.0 μm will be excluded from the detection and analysis.

(b) sensi: defines "circularity" for detecting nuclei. Circularity determines the sensitivity of the function "imfindcircles" embedded in the custom-made function "DetectNucleus" (line 87). The preset is 0.935 which is optimized for *C. elegans* germ cell nuclei.

(c) radius: defines the radius of a spherical region of interest (ROI) to count the number of mRNAs within the region. The center of ROI is placed on each nuclear center to estimate the number of mRNAs in vicinity of each nucleus. The preset is 2.5 (μm) which is roughly the radius of *C. elegans* germ cells in the distal gonad.

(d) vLim: defines the maximum radius of the 3D Voronoi cell, which estimates the boundary of each cell based on the 3D location of all nuclei. The number of mRNA is counted in each Voronoi cell and recorded as the total number of mRNA in each cell. The preset is 3 (μm) which is a typical radius of *C. elegans* germ cells located next to the rachis in the distal gonad.

6. All information pertaining to detected RNA and nuclear spots along with the image information are stored in the variable "af." The specific information saved in each column of "af" is annotated in the main script (lines 593–649). This information can be used for further analyses as shown in our previous work [21, 22].

3.2 MS2 RNA Live Imaging

3.2.1 Sample Preparation for Imaging

1. A gel-pad microscope slide is required for live *C. elegans* imaging. The method to prepare the gel-pad slide was modified from [24, 36] (*see* **Note 19**). To make the gel-pad slide, melt the 5% agarose/M9 (w/v) solution using a microwave or a heat block and put 300–500 μL solution onto the middle of a microscope slide using P-1000 pipette. Cut the tip of the pipette tip to make a larger opening for better flow of the agarose solution if needed. Immediately put another microscope slide on top of the microscope slide with agarose drop. Remove the top or bottom slide after a few seconds. The 5% agarose solution can be premade and reheated when the experiment is conducted.
2. Add 0.5–2 μL polystyrene microbead suspension onto the middle of the gel-pad (*see* **Note 20**).
3. Immediately add 0.5–2 μL of freshly made 50 mM serotonin solution (dissolved in M9) to bring its final concentration to 25 mM. Use the same volume of serotonin solution as the microbeads (*see* **Note 21**).
4. Move the gel-pad slide to the dissecting scope and pick 10–15 well-fed worms using a worm pick and OP50. Gently move worms in the serotonin solution on the gel-pad. Do not gouge the gel-pad with a worm pick. Use the surface tension of the serotonin solution to detach worms from the worm pick. Additional OP50 can be added around the edges of serotonin solution drop on the gel-pad.

5. Gently place the cover glass on the gel-pad. Do not move the cover glass once it sits on the gel-pad as moving it can kink, twist, or sever the worms.
6. Check the worms on the microscope to confirm they are immobilized. If worms still move, prepare a new gel-pad slide and repeat the procedure with a smaller volume of microbeads and serotonin. Make sure the pharynx of the worm on the microscope slide is pumping at a normal rate (*see* **Note 22**).
7. Apply VALAP (*see* Subheading 2) around edges of the cover glass to completely seal the slide. Failing to do so will result in a dried, wrinkled gel-pad.
8. The slide can be attached to a temperature controlling system such as CherryTemp and mounted on the microscope stage with a chip holder to keep a constant temperature throughout the live imaging process or to shift temperature during the experiment (*see* **Note 23**).

3.2.2 MS2 Imaging

Live imaging was performed immediately after making the gel-pad microscope slide. Both confocal and wide-field microscopes worked well for *sygl-1* RNA live imaging [24] (unpublished for the wide-field system). Here, we describe our image acquisition settings for both imaging systems (*see* **Note 24**).

Confocal Microscopy Settings

1. Microscope: Leica SP8 Confocal Laser Scanner.
2. Objective lens: Leica HC PL APO CS2 63×/1.40 OIL.
3. Photon detector: Sensitive hybrid detector (HyD).
4. Frequency: 900–1000 Hz.
5. Zoom factor: 2.5×.
6. Image size: 512 × 512 or 1024 × 512.
7. Pinhole: 95.5–105.1 μm (1–1.5 airy unit).
8. Laser power: 0.3–0.4% Argon laser (488 nm) for GFP and 0.6–1% HeNe laser (594 nm) for mCherry.
9. The signal acquisition bandwidth: 50 nm. Start the acquisition window 5–10 nm from the end of the excitation laser range. For example, GFP was excited with the 488 nm laser and the emission was acquired at 496–546 nm. No line/frame averaging or accumulation was used.
10. Gain: 40.
11. *Z* step size: 0.4 μm.
12. Optional: Autofocusing was enabled for every other time point with 594-nm Argon laser to keep the gonad that is imaged in the similar position on the *z*-axis.

Wide-Field Microscopy Settings

1. Microscope: Leica THUNDER Imager 3D Assay with RFID and LED8 (DMi8).
2. Objective lens: Leica HC PL APO 63×/1.40–0.60 OIL.
3. Filter set: DFT51011 quad cube.
4. LED Light intensity: 10–30%, typically 20%.
5. Exposure time: 50–100 ms, typically 50 ms.
6. *Z* step size: 0.4 μm.

3.2.3 MS2 Image Analysis

Even if the worm on a gel-pad slide is completely immobilized, the gonad inside the worm being imaged can move or twitch time to time due to the movement of the intestine, muscles, or embryos. This results in shift, drifting or fluctuation of gonads in the MS2 movies. Theses gonads can be aligned in two steps using our customized, automated ImageJ macros, which are available at https://github.com/chlasic/MS2_analysis.

1. Open ImageJ or Fiji and open the first macro "MultiStackReg_BatchProcess1of2.ijm." We use the shortcut "["to open a script window to open the macro (*see* **Note 25**).
2. Click "Run" on the bottom of the window to start the macro. It lets users choose two directories, one for images to be processed and the other for saving processed images. Then, it will process all images in the folder and save the results in the designated folder location.
3. Open the second macro "MultiStackReg_BatchProcess2of2.ijm" and click "Run." It will also ask two directories as above and will process all images (*see* **Note 25**).
4. The user can open the final images to check if all *z* planes are aligned throughout the time lapse.
5. If the automated alignment does not work properly, the user can manually align one time point using "TranslateStack.ijm" or multiple time points using "TranslateStackAllAfterPos.ijm."
6. Split all gonadal images (lif or tif files) in two halves on the *z*-axis in ImageJ to minimize overlap of germ cell nuclei for further analysis. Use "duplicate" function to make two hyperstacks, each containing a half of the *z*-stack image.
7. *Z*-project each half using sum slices.
8. Draw a circular ROI (diameter of 1 μm) around each *sygl-1* MCP::GFP dot in the *z*-projected images to measure signal intensity, which estimates the number of nascent transcripts at the locus. Manually follow the individual dots and record their signal intensity throughout the MS2 movies (*see* **Note 26**). The same ROI should be used to measure background, which is drawn inside the gonad where there is no MS2 signal. The

recorded signals can be further analyzed such as spatiotemporal pattern analysis of transcriptional burst duration or frequency as in our previous publications [24].

4 Notes

1. Regular (non–RNase-free) reagents and consumables such as tubes and pipette tips can be used until the end of sample fixation with formaldehyde. Thereafter, all reagents must be RNase-free. We wipe the bench and pipettes with RNaseZap and use RNase-free microcentrifuge tubes, filtered nuclease-free pipette tips. Unopened packages of non–RNase-free microcentrifuge tubes can also be used throughout the entire protocol if they are kept separately and handled cleanly. Use PPE at all times to protect the sample from RNase contamination.
2. Dextran sulfate, E. coli tRNA, and nuclease-free BSA can be excluded from the hybridization buffer. They help reduce overall background of smFISH images. Leave them out when making the hybridization buffer if smFISH signal is too weak even at the highest probe concentration.
3. We designed the smFISH probes using "Stellaris probe designer" (https://www.biosearchtech.com/support/tools/design-software/stellaris-probe-designer) and ordered from Biosearch Technologies (https://www.biosearchtech.com/). Available fluorescence dyes are listed here (https://www.biosearchtech.com/support/education/stellaris-rna-fish/dyes-and-modifications-for-stellaris). Carefully choose the dyes that are compatible with the microscope system used for the experiment. Choose the dyes that have the least overlapping spectra when multiplexing the probes to eliminate bleed-through between channels. We recommend empirically testing emission collection windows for bleed-through on confocal microscopes. For example, we found that exciting with a 561 nm laser and collecting wavelengths greater than 588 nm could detect CAL Fluor Red 610 signal when multiplexed, so we limited our Quasar 570 emission collection to 564–588 nm. For multiplexing probes, we used two among Quasar 570, CAL Fluor Red 610 and Quasar 670 for the Leica confocal microscope, and TAMRA and Quasar 670 for the Leica Thunder wide-field microscope. We confirmed specificity of the probes by conducting BLAST/BLAT search with the *C. elegans* whole genome sequence (https://wormbase.org//tools/blast_blat). Only probes that match to the target sequence were used.

4. The master smFISH probe stock solution is at 125 μM (5 nmol probes in 40 μL TE buffer). We dilute the master stock 1:20 in TE buffer (6.25 μM) to make a personal working probe stock. We dilute this working probe stock 1:50 in hybridization buffer (125 nM) during the smFISH experiment. Try different dilutions of the working stock to optimize the smFISH signal. For example, try a 1:10 working stock (final concentration: 250 nM) or 1:5 (500 nM) dilution if smFISH signal is too dim. In case of high background, try 1:50 (50 nM) or 1:100 (25 nM).
5. During worm dissection, use 2 mL PBST to wash one 60-mm NGM plate. You will be able to pipette up about 1.5–1.7 mL with worm suspension from the plate. In case of using 100-mm glass dish for dissection, we use 10 mL worm suspension containing 50–100 worms.
6. For worm dissection, finishing worm dissection within 20 min ensures preservation of an intact gonad. After 30 min of levamisole treatment, you may notice deformation of gonad.
7. Periodically rewipe gloves and pipettors with RNaseZap.
8. During and after smFISH probe hybridization, minimize exposure of the sample to light. Shield tube in a cover (your hand, aluminum foil fold, cardboard box tent, etc.) while changing buffers or tips. We always wrap the tubes containing the sample and smFISH probes in foil. To remove liquid from samples, use a P-200 or P-1000 without exposing tube to too much incident light such as the base light of the dissecting scope.
9. For smFISH probe hybridization, you can leave samples for up to 72 h. Longer incubation may improve smFISH signal.
10. **Steps 3–4**, Subheading "Sample Washing and Mounting (Day 3)" can be skipped for imaging with the confocal microscopy. The additional washes help with background when using widefield microscopy.
11. For mounting smFISH samples on a microscope slide, remove as much wash buffer as possible from fixed samples. Resuspend gonads in 10–20 μL PLG depending on # of dissected worms, then collect 5–10 μL using a pipette and drop 8–10 small droplets around the center of microscope slide. Microscope slides and coverslips can be baked as an extra precaution against RNase contamination, or simply dedicate a slide box to smFISH. After mounting sample, use a P10 pipette tip or a worm pick to carefully reposition gonads if needed.
12. After mounting sample on a microscope slide, do not press the cover glass hard, which can rupture samples. Do not try to remove all small bubbles in the slide which can crack the cover glass or break the samples. Adding some extra ProLong Gold

to the sample can help removing bubbles on the slide. This also dilutes the residual wash buffer which can improve the smFISH signal.

13. We follow the ProLong Gold manual's recommendation to cure 24–60 h, but the sample slides can be cured for as little as 2 h before imaging. Longer curing times (48–60 h) improve smFISH detection on our hands. The ProLong manual also includes recommendations for cure times and optimal mounting medium for different fluorophores (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0002469_ProLong_SlowFade_Antifade_Mountants_PI.pdf).
14. The smFISH microscope slides are generally good for at least 6 months from mounting when they are completely sealed with nail polish.
15. The MATLAB code for image processing reads image files along with their metadata, detects objects that fall in a particular category (e.g., the tissue outline, RNAs and nuclei), and records the information associated with objects (e.g., 3D coordinates, integrated intensities, and shape).
16. For image processing with the MATLAB code, a lower threshold allows detection of dimmer spots, which usually have lower signal-to-noise ratio, on microscope images.
17. Choosing the right threshold in the MATLAB code is critical to accurate RNA or nucleus detection but it can be time consuming. A good practice is to pick a portion of images (5–10 images) and use them to optimize the thresholds. The random number generator "randi" function in MATLAB can be used for unbiased image choices. The selected threshold typically works well for most of images with the same image acquisition settings.
18. The MATLAB code and the workflow described here can be used for detecting transcripts of interest in other tissues such as intestine, seam cells, and neurons in *C. elegans* as well as other model systems such as *S. cerevisiae*, *Neurospora*, and tissue culture (unpublished, confirmed by personal conversations). The parameters "nrange," "sensi," "radius," and "vLim" should be empirically determined for accurate detection and analysis with systems other than the *C. elegans* germline. For "sensi," a higher value will make the detection more stringent and exclude nuclear objects with relatively irregular circular shapes. A lower value will allow detection of even less-circular objects. Three methods are used to count mRNAs around each nucleus: (A) spherical ROIs with a user-set "radius," (B) 3D Voronoi cells without any limit, which can include the rachis in the distal gonad, and (C) 3D Voronoi cells with a limit for cell radius of "vLim". The mRNA counts from each of the three

methods are stored in the variables "nuc" and "af" (see the annotation in lines 325–328 of the main script) so that the user can choose an mRNA estimate that best represents the number of mRNA per cell for the type and shape of the cells used for smFISH. Method A is particularly useful for complete syncytial systems with no individual cell boundaries as it will consider which nucleus (or nuclei) the mRNAs belong to based only on their proximity to the nucleus (or nuclei) within the user-defined "radius". If two or more ROIs overlap, the number of mRNAs within the overlapped region will be divided by the number of overlapping ROIs and equally assigned to these ROIs because there is an equal chance these mRNAs belong to those ROIs in syncytium. In contrast, methods B and C are useful for systems with separate cells. They draw theoretical cell outlines around each nucleus and record the number of mRNAs within each Voronoi cell. Method B is typically for tissue with no spaces or gaps between individual cells and C is for tissue with gaps which can be excluded from mRNA counting by setting "vLim". We used 3D Voronoi cells with a limit to estimate the number of mRNAs per cell [21, 22].

19. The thickness of gel-pad is important for worm immobilization and long-term live imaging. 1.5–2 mm (four layers of time tape) thick gel-pad gave us the best result, keeping normal pharyngeal movement (3–5/s) for at least several hours. Thinner gel-pad can help immobilize worms but the pharynx tends to stop after a few hours of imaging (1–3 h). It was harder to keep worms immobilized with a gel-pad thicker than 2 mm.
20. To immobilize the worm, we have also tried 0.05-μm polystyrene microbeads which gave us a similar result for worms at L4 stage or older. We speculate the smaller microbead may better immobilize smaller worms (L1–L3).
21. After adding microbeads and serotonin, worms should be immediately and quickly loaded on the gel-pad with minimum exposure to any direct light such as the base light for the dissecting microscope. The solution on the gel-pad dries up quickly, which makes harder to load worms on it.
22. Healthy worms on the gel-pad slide have a pharyngeal pumping rate of 3–5/s and an egg laying rate of 2–5/h, and a mitotic index of about 6.25, consistent with worms on nematode growth (NGM) plates.
23. The consistent temperature helps keep worms alive for extended time. We used CherryTemp but any temperature controlling system can be used.
24. The multipoint imaging can be used for monitoring several worms on the same slide over time. Make sure to use autofocusing to minimize image drifting on the *z* axis.

25. The image processing macro "MultiStackReg_BatchProcess1of2.ijm" aligns gonadal images along the *z*-axis at each timepoint. "MultiStackReg_BatchProcess2of2.ijm" uses the processed images from the first macro to further align them throughout the MS2 movie.
26. Most MCP dots did not move dramatically inside the nucleus in the *C. elegans* germ cells (95%), which made it easier to trace transcriptional bursting [24]. Other systems might exhibit more dynamic chromosomal movement.

Acknowledgements

CHL was supported by the American Heart Association (18POST34030263). TRL was supported by the National Science Foundation Graduate Research Fellowship Program (Grant No. DGE1256259). JK is grateful to NIH R01 GM134119 for support.

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