Journal of Visualized Experiments

# Probing mRNA kinetics in space and time in Escherichia coli using two-color single-molecule fluorescence in situ hybridization

--Manuscript Draft--

| | |
|---|---|
| **Article Type:** | Invited Methods Article - JoVE Produced Video |
| **Manuscript Number:** | JoVE61520R2 |
| **Full Title:** | Probing mRNA kinetics in space and time in Escherichia coli using two-color single-molecule fluorescence in situ hybridization |
| **Section/Category:** | JoVE Biology |
| **Keywords:** | smFISH; RNA imaging; mRNA localization; single cell; single molecule; Transcription; transcription elongation; premature termination; mRNA degradation; mRNA lifetime. |
| **Corresponding Author:** | Sangjin Kim<br>University of Illinois at Urbana-Champaign<br>Urbana, IL UNITED STATES |
| **Corresponding Author's Institution:** | University of Illinois at Urbana-Champaign |
| **Corresponding Author E-Mail:** | sangjin@illinois.edu |
| **Order of Authors:** | Sangjin Kim |
| | Kavya Vaidya |
| **Additional Information:** | |
| **Question** | **Response** |
| Please indicate whether this article will be Standard Access or Open Access. | Open Access (US$4,200) |
| Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations. | Urbana, Illinois, USA |

**TITLE:**

# Probing mRNA Kinetics in Space and Time in *Escherichia coli* Using Two-Color Single-Molecule Fluorescence In Situ Hybridization

**AUTHORS AND AFFILIATION:**

Sangjin Kim[1,2,3,4], Kavya Vaidya[1,2]

[1]Department of Physics, University of Illinois at Urbana-Champaign, Urbana, IL, USA
[2]Center for the Physics of Living Cells, University of Illinois at Urbana-Champaign, Urbana, IL, USA
[3]Carl R. Woese Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, IL, USA
[4]Center for Biophysics and Quantitative Biology, University of Illinois at Urbana-Champaign, Urbana, IL, USA

Corresponding author
Sangjin Kim (sangjin@illinois.edu)

Email Address of Co-Author:
Kavya Vaidya (kvaidya2@illinois.edu)

**KEYWORDS:**

smFISH, RNA imaging, mRNA localization, single cell, single molecule, transcription, transcription elongation, premature termination, mRNA degradation

**SUMMARY:**

This protocol describes an application of single-molecule fluorescence in situ hybridization (smFISH) to measure the in vivo kinetics of mRNA synthesis and degradation.

**ABSTRACT:**

Single-molecule fluorescence in situ hybridization (smFISH) allows for counting the absolute number of mRNAs in individual cells. Here, we describe an application of smFISH to measure the rates of transcription and mRNA degradation in *Escherichia coli*. As smFISH is based on fixed cells, we perform smFISH at multiple time points during a time-course experiment, i.e., when cells are undergoing synchronized changes upon induction or repression of gene expression. At each time point, sub-regions of an mRNA are spectrally distinguished to probe transcription elongation and premature termination. The outcome of this protocol also allows for analyzing intracellular localization of mRNAs and heterogeneity in mRNA copy numbers among cells. Using this protocol many samples (~50) can be processed within 8 h, like the amount of time needed for just a few samples. We discuss how to apply this protocol to study the transcription and degradation kinetics of different mRNAs in bacterial cells.

**INTRODUCTION:**

The flow of genetic information from DNA to mRNA and protein is one of the most fundamental cellular processes, whose regulation is important for cellular fitness[1]. The number of mRNAs in a cell is determined by two dynamic processes, transcription, and mRNA degradation. However, how transcription and mRNA degradation are regulated in time and space of a single cell remains not completely understood, largely due to the shortage of experimental methods to quantitatively probe them in vivo.

Methods based on total mRNAs extracted from a population of cells, such as Northern blot, RT-PCR, RNA sequencing, and gene expression microarrays, can measure the relative difference in mRNA levels and have been widely used to analyze the rate of transcription elongation[2-5] or the rate of mRNA degradation[6,7]. However, they do not provide the absolute number of mRNAs per cell, and hence, they are not suitable for probing the rate of transcription initiation[8]. Also, because mRNAs are extracted from a population of cells, the spatial distribution of mRNAs within a single cell and the variability of mRNA copy numbers among cells cannot be measured.

Next-generation RNA sequencing on individual cells (scRNAseq) can quantify the number of mRNAs per cell in a genomic scale[9]. However, it remains difficult to use this technique to measure transcription kinetics, due to challenges with sample preparation and high cost. In particular, the application of scRNAseq to bacteria has been technically difficult due to low mRNA abundance[10,11].

Single-molecule fluorescence in situ hybridization (smFISH) is based on the hybridization of fluorescently-labeled single-stranded probes whose sequences are complementary to the target mRNA of interest[12,13]. The concept of sequence-specific hybridization is similar to that used in Northern blot or RT-PCR, but the hybridization is done in situ within fixed cells, to preserve the native localization of mRNAs. The signal of a single mRNA is amplified using many probes, ~20 nucleotides (nt) in length, hybridizing to different parts of an mRNA (**Figure 1A**)[13]. In this "tiling" probe approach, the number of probes needed to detect a single mRNA sets a lower limit on the length of mRNA that can be assayed. Alternatively, the mRNA of interest may be transcriptionally fused to a non-coding array of tandem Lac operator sequences, such that multiple copies of a fluorescently labeled lacO probe label a single mRNA (**Figure 1B**)[14].

smFISH has been used to quantify the number of mRNAs per cell at steady state (i.e., when synthesis and decay are in balance) and to analyze the mean and variability of mRNAs among bacterial cells[15-17]. Recently, smFISH has been applied to quantify mRNA numbers at non-steady state, right after induction or repression of gene expression in *E. coli*[18-20]. The temporal changes in the absolute mRNA copy numbers were then used to calculate the rate of transcription initiation, elongation, and termination, as well as the rate of mRNA degradation. For this application, conventional smFISH procedures can be cumbersome because there are many samples, each representing one time point, that need to go through multiple buffer exchange steps (i.e., centrifugation and washing). Here, we describe an smFISH protocol, in which the sample handling steps are dramatically simplified by having cells adhered to the surface of a coverslip and by aspirating liquids with a vacuum filtration system[14,19]. Using the expression of

*lacZ* in *E. coli* as an example, the full workflow (**Figure 2**) is demonstrated, including image analysis (**Figure 3**) yielding the in vivo kinetics of transcription (initiation, elongation, and termination) and mRNA degradation, cell-to-cell variability in mRNA expression, and mRNA localization. We anticipate that the protocol is widely applicable to probe in vivo kinetics and localization of other mRNAs in various bacteria species.

**PROTOCOL:**

## 1. Preparation of smFISH probes

NOTE: To label smFISH probes with a single fluorophore, follow a standard protocol for labeling nucleic acid oligonucleotides based on NHS ester chemistry[21].

1.1. Design smFISH probes. Decide whether to use "tiling" probes or "array" probes (**Figure 1**) for the gene of interest. See the **Discussion** section on how to make the decision.

1.1.1. For "tiling" probes (**Figure 1A**), use an online probe designer tool (e.g., see **Table of Materials**).

1.1.2. For "array" probes (**Figure 1B**), perform a BLAST sequence search to make sure that the probe sequence is not complementary to any other mRNA sequences.

1.1.3. To study *lacZ* mRNA transcription and degradation kinetics, use two sets of 24 probes, each set covering the first and last 1 kb regions of *lacZ* (3,072 bp)[19].

NOTE: These probe sets are, hereinafter, referred to as "5' mRNA probe" and "3' mRNA probe", respectively. Sequences of these probes are listed in the **Table of Materials**.

1.2. Order probe sequences as DNA oligonucleotides with a C6 amino linker at the 5' end. Dissolve individual probes in water to 1 mM.

1.3. Combine equimolar amounts of probes for "5' mRNA probe" and "3' mRNA probe" sets. For example, for the 5' mRNA probe set for *lacZ*, combine 20 µL of each probe (total 24 kinds of probes in the set).

1.4. Perform ethanol precipitation[22] of the combined probes to remove any contaminations of primary and secondary amines (such as Tris, glycine, and ammonium salts) that can inhibit the conjugation reaction. In the end, dissolve the DNA pellet in 100 µL of water (yielding ~4.5 mM of DNA in a probe set).

NOTE: This step is recommended even if the probes underwent a standard desalt purification by the manufacturer. A standard filter-based purification may work in place of and in addition to the ethanol precipitation.

1.5. Choose two spectrally distinct fluorophores with a monofunctional NHS ester moiety, such that 5' and 3' mRNA probe sets can be labeled differentially. For example, prepare Cy5 NHS ester for 5' mRNA probes and Cy3B NHS ester for 3' mRNA probes. Dissolve each type of fluorophores in anhydrous DMSO to final 20 mg/mL (~25 mM).

1.6. Prepare 0.1 M sodium bicarbonate (pH 8.5) right before each labeling reaction. Exposure to air for a long time will lower its pH and reduce the labeling efficiency.

1.7. For the conjugation reaction, combine the following: 15 µL of the Cy5 fluorophore stock (from Step 1.5), 4 µL of 5' mRNA probe set (from Step 1.4), 75 µL of sodium bicarbonate (from Step 1.6), and 7 µL of water. Wrap the tube with aluminum foil and shake at room temperature for 3-6 h.

NOTE: Longer incubation does not necessarily result in greater labeling efficiency. Also, the reaction can be scaled up or down if the concentrations of the components are maintained.

1.8. Repeat the above step for the 3' mRNA probe set and the corresponding fluorophore (i.e., Cy3B NHS-ester).

1.9. Perform ethanol precipitation[22] to remove un-reacted dye molecules. Dissolve the pellet in water (~50 µL).

1.10. Estimate the concentrations of DNA and fluorophore by using a UV-Vis spectrometer.

1.10.1. Measure the absorbance at 260 nm and 559 nm (Cy3B) or 649 nm (Cy5). If the sample is too concentrated to yield an accurate measurement, dilute 1 µL of the sample to 10 µL.

1.10.2. Convert the absorbance to the concentration:

$$[\text{DNA}] = \frac{(abs\ at\ 260\ nm) * (dilution\ factor)}{\varepsilon_{DNA}}$$

$$[\text{Cy5}] = \frac{(abs\ at\ 649\ nm) * (dilution\ factor)}{\varepsilon_{Cy5}}$$

$$[\text{Cy3B}] = \frac{(abs\ at\ 559\ nm) * (dilution\ factor)}{\varepsilon_{Cy3B}}$$

$\varepsilon_{DNA}$ = 0.2 µM$^{-1}$ (for 20-nt single-stranded DNA), $\varepsilon_{Cy5}$ = 0.25 µM$^{-1}$, and $\varepsilon_{Cy3B}$ = 0.13 µM$^{-1}$

NOTE: [DNA] is the concentration of total probes within the solution. The concentration of individual probes is about 24x lower. The concentration of total probes will be used as "probe concentrations" from this point. If the ratio between [DNA] and [dye] is 1, the following HPLC step may be skipped[23], and the sample should be diluted in TE buffer (10mM Tris-HCl pH 8.0 with 1mM EDTA) to final 4-5 µM for storage.

1.11. (Recommended) Purify the labeled probes from unlabeled probes and free dyes by

using HPLC.

NOTE: Although this additional purification step will lead to the loss of sample, it is beneficial for the downstream applications. Removal of unlabeled DNA probes will increase the fluorescence signal from mRNA targets and removal of unreacted dyes will reduce background fluorescence.

1.11.1. Prepare HPLC with a standard analytical C18 column, 0.1 M triethylammonium acetate (TEAA) as buffer A, and acetonitrile as buffer B.

1.11.2. Add 1 M TEAA to the sample (~50 µL from Step 1.9) to make 0.1 M TEAA. Filter the sample using a syringe filter.

1.11.3. Set the gradient program as follows: 0-5 min with 0% B, 5-35 min with a 0-30% linear gradient of B, 35-37 min with a 30-100% linear gradient of B, and 37-40 min with 0% B. Keep the flow rate at 0.1 mL/min and record chromatograms at 260 and 649 nm (for Cy5 labeling) or at 260 and 559 nm (for Cy3B labeling).

1.11.4. Collect the eluted sample when the absorbance increases in both DNA and fluorophore channels.

1.11.5. Concentrate the purified probe sample using a vacuum concentrator and re-suspend the pellet in 50-100 µL TE buffer.

1.12. Check the concentration of DNA and fluorophore by using a UV-Vis spectrometer (see Step 1.10). Dilute, if necessary, to make final concentration around 4-5 µM.

## 2. Preparation of solutions

2.1. Prepare a large volume of DEPC-treated water and buffers (**Table 1**). These solutions can last over a year at room temperature.

2.2. Prepare 4x fixing solution and wash solution (**Table 1**).

2.3. Prepare the pre-hybridization solution and probe hybridization solution (**Table 1**). Prepare the probe hybridization solution during incubation in Step 5.1 or Step 6.1 and then keep the solution in a 37 °C countertop shaker for 20-40 min (cover to minimize exposure to light).

NOTE: The concentrations of formamide, SSC, and probe were optimized for the *lacZ* probe sets to minimize background fluorescence while maximizing the real signal. See the **Discussion** section for details on how to modify these concentrations for different applications.

## 3. Preparation of coverslips and glass slides

3.1. Clean coverslips and glass slides.

3.1.1. Place individual coverslips and slides in a Coplin jar using forceps. Ensure that the coverslips and slides are separated and not touching each other.

3.1.2. Fill the jar with 100% ethanol and close the lid. Place the jar in a water-bath ultrasonic cleaner and sonicate for 15-20 min.

NOTE: For the water-bath sonicator, it is recommended to turn off the heater function.

3.1.3. Pour out ethanol and wash with ultrapure water 3-4x. Use water flowing directly from the water purification machine.

3.1.4. Pour out the water from the jar and fill it with 70% ethanol. Close the lid and perform sonication for 15-20 min and wash with ultrapure water.

3.1.5. Fill the jar with ultrapure water and sonicate for 15-20 min.

NOTE: Coverslips and glass slides can be kept overnight in the Coplin jar filled with ultrapure water.

3.1.6. Take a slide or a coverslip out of the Coplin jar using clean forceps and blow-dry it using $N_2$ gas. Repeat this for the remaining slides and coverslips.

3.2. Place the dried slides in a clean storage box until use in Step 7.4. Place the dried coverslips in an empty 1,000-µL pipette tip box, which will serve as a "chamber" in the remaining procedure.

3.3. Using a hydrophobic marker, draw circles on the coverslips following circular holes in the pipette tip box. These circles (~0.5 cm in diameter) will serve as "wells". Wait at least 5-10 min for the marker to be completely dried.

NOTE: Always keep the lid of the tip box closed.

3.4. Apply a 20 µL drop of 0.1% poly-L-lysine to each well. Incubate for 10-50 min at room temperature.

NOTE: Adjust this volume according to the well size. Ensure that the solution completely covers the well area. For longer incubation, be careful to avoid evaporation.

3.5. After incubation aspirate poly-L-lysine without touching the surface as this will scrape the poly-L-lysine off. Then apply a drop (~20 µL) of DEPC water to the poly-L-lysine treated wells. Close the lid of the "chamber" to prevent evaporation until Step 5.1.

## 4. Time-course experiment and sample fixation

4.1. Grow *E. coli* cells in ~20 mL liquid culture in a 250-mL flask. Keep the flask in a water bath shaker (30 °C) and continue shaking. Stop the shaker only when taking samples.

NOTE: Results presented in this paper are obtained from MG1655 culture grown in M9 minimal medium supplemented with 0.2% glycerol, 0.1% casamino acids, and 1 mg/L thiamine to an exponential growth phase ($OD_{600}$~0.2).

4.2. Add 250 µL of the 4x fixing solution in an empty 1.5 mL tube. Repeat and prepare multiple tubes, as many as the time points to be taken. Label the tubes with time point numbers and keep them at room temperature.

4.3. Take 750 µL of cell culture ($OD_{600}$~0.2) before starting a time-course experiment. Add the culture to a tube marked for "time zero" (from Step 4.2). Invert the tube gently to mix cells with the fixing solution.

NOTE: Do not pipette up and down to mix, vortex, or "be rough" on the cells. This sample represents the repressed state and will be used as a control to calculate the fluorescence intensity of a single mRNA (see Step 9.4).

4.4. Add 0.02-1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) to the liquid culture to induce *lacZ* expression. Start a timer at this point (t = 0 min) and sample at a certain time interval (e.g., every 1 min) from then on. For sampling, repeat Step 4.3.

4.5. Add 5 mM orthonitropheynl-β-D-fucopyranoside (ONPF) or 500 mM glucose[24] at a certain time during the time-course experiment (e.g. at t = 1.5 min) to repress *lacZ* expression. After re-repression, continue to sample the cultures (Step 4.3) to track mRNA degradation.

NOTE: Repression can also be done with ~400 µg/mL rifampicin, a transcription initiation inhibitor[25].

4.6. For fixation, incubate the tubes containing sampled cells at room temperature for 15 min, followed by incubation in ice for 30 min.

4.7. To remove fixatives, centrifuge the tubes at 4,500 *x g* for 4 min at room temperature. Remove the supernatant with a pipette.

NOTE: Be sure to discard formaldehyde in a separate waste container following the safety protocol.

4.8. Add 1 mL DEPC-PBS and re-suspend the cells. Repeat centrifugation and re-suspension 2x more times.

NOTE: Fixed cells are fragile and need gentle treatment. Carefully re-suspend the pellet and avoid bubbles.

4.9. After the final wash step, re-suspend cells in ~30 µL DEPC-PBS.

## 5. Permeabilization of cell membranes

5.1. Apply each time point sample to different wells on the coverslip (~30 µL per well). Wait for 10-30 min at room temperature for cells to adhere on the surface. Avoid merging of the liquid drops between wells.

5.2. To rinse off unbound cells, aspirate the liquid and apply ~20 µL DEPC PBS to each well. Aspirate DEPC PBS within a few minutes.

5.3. Permeabilize the cell membranes by applying 15 µL of 70% ethanol to each well for 4 min. Aspirate the ethanol after the 4 min, and make sure that the wells are completely dry.

NOTE: It is critical to limit the ethanol treatment for 4-5 min. Longer treatment will result in over-permeabilization.

5.4. Apply 30 µL of the wash solution to each well.

## 6. Probe hybridization

6.1. Aspirate the wash solution from each well. Apply 30 µL of the pre-hybridization solution to each well. Incubate the chamber in the 37 °C oven for 30 min.

NOTE: Add ~50 mL of water to the bottom of the chamber for humidity. Keep the water for the remaining steps.

6.2. Aspirate the pre-hybridization solution from each well. Apply ~30 µL of the probe hybridization solution to each well. Cover the chamber with aluminum foil and incubate in the 37 °C oven for 2 h.

NOTE: Make sure that the probe hybridization solution is in the 37 °C countertop shaker before this step. Avoid the merging of liquids between wells. Apply a smaller volume of the solution to each well, if needed.

## 7. Post-hybridization wash and preparation for imaging

7.1. Using a multichannel pipette, apply ~30 µL of the wash solution to each well all at once. Aspirate and repeat 3-5x times of washing. Incubate the chamber in the 37 °C oven for 15-30 min.

7.2. Repeat Step 7.1 two more times.

7.3. Wash each well with DEPC-PBS 5x times. Follow the method used in Step 7.1 but skip 37 °C incubation.

7.4. Aspirate the liquid from the coverslip. Apply 4 µL of DEPC-PBS to each well.

7.5. Using forceps, lift the coverslip, flip, and gently place over a glass slide (from Step 3.2). Avoid bubbles.

7.6. Seal the edges of the coverslip with silicone dental gum.

7.7. Wait until the gum is solidified. One can pause here and store the slide overnight at 4 °C.

NOTE: Other smFISH protocols suggest adding oxygen scavenging reagents (e.g., glucose oxidase/catalase) or using a commercial anti-fade mounting medium[14,26] to increase the photostability of the fluorophores.

## 8. Imaging

8.1. To find an area of interest, use the live mode of phase contrast imaging. Change the field of view within a well by maneuvering the stage joystick. Choose an area where the cell density is optimal (i.e., there are many cells that are mostly separated). Adjust z-focus such that phase-contrast cell images are in focus.

8.2. Take snapshots in the order of Cy5 (4-s exposure), Cy3 (2-s exposure), and phase contrast (0.2-s exposure).

8.3. Repeat Steps 8.1-8.2 to acquire images of ~10 different areas within a well.

8.4. Move the objective to another well and repeat Steps 8.1-8.3.

8.5. Export images as TIFF files.

8.6. (Optional) Image multi-color beads adsorbed on the coverslip surface in Cy5 and Cy3 channels to determine spatial shift between Cy5 and Cy3 channels for image registration purposes.

8.6.1. Apply ~10 µL of multi-color fluorescent beads (0.2 µm diameter) on a clean coverslip surface and wait for 10-30 min. After washing with ~50 µL of PBS, apply ~5 µL of PBS and sandwich the coverslip with a glass slide. Seal and mount on the microscope.

8.6.2. Image beads in both Cy5 and Cy3 channels.

## 9. Image analysis

NOTE: Matlab code used in this step is available in the following GitHub website: https://github.com/sjkimlab/Code_Publication/tree/master/JoVE_2020. The GitHub folder contains everything needed for the image analysis, including parameter values for cell segmentation and spot identification. The procedure in this step is further explained in the master script, called "FISHworkflow.m".

9.1. Open a cell segmentation tool, such as microbeTracker[27] or Oufti[28], and load phase contrast images. Choose "Independent frames" and press a button called "All frames" to begin the segmentation process, from which cells are identified and their contours are calculated (**Figure 3B**,**C**).

NOTE: Detailed protocols for using these software packages are available online (e.g., oufti.org).

9.2. Load Cy5 fluorescence images in the spotFinder function of microbeTracker or Oufti, and press the "**Run**" button to begin spot identification and quantification based on 2D Gaussian fitting (**Figure 3B**,**C**). Repeat this step for Cy3 fluorescence images to analyze spots in the Cy3 channel. This step produces a list of spots in each cell, including their intensities and coordinates.

9.3. (Optional) Filter out dim spots (false positives) using a threshold, as explained in in the FISHworkflow.m file.

NOTE: Examine fluorescent spots in the negative control (e.g. MG1655 Δ*lacZ*) and determine the threshold to filter out false positives.

9.4. To obtain the spot intensity of a single mRNA, use a list of spot intensities measured at time zero (before adding IPTG), and fit the distribution of spot intensities with a Gaussian mixture model with two mixture. Take the peak position of the first Gaussian population (black line in **Figure 3D**-**3E**) as the spot intensity of a single mRNA. Perform this for Cy5 spots and Cy3 spots separately to obtain the spot intensity of a single 5' and 3' *lacZ* mRNA.

NOTE: Repeat this in every time-course experiment because the peak positions can vary slightly in different experiments. The spot intensity of a single mRNA (either 5' or 3' mRNA) is used as a normalization factor to convert spot intensities to the number of mRNAs in the next step.

9.5. Divide the fluorescence intensity of a spot with the intensity of a single mRNA (from Step 9.4) to obtain the number of mRNAs within a spot. Sum normalized spot intensities within a cell to calculate the total number of mRNA in a cell (**Figure 3F**). Perform these calculations for 5' and 3' mRNA separately.

9.6. Calculate and plot the mean mRNA numbers per cell at each time point (e.g., **Figure 4B**),

and analyze the in vivo kinetics of transcription and mRNA degradation from the temporal change in the mean mRNA levels (**Figure 4B**).

9.6.1. To obtain the rate of transcription elongation, perform a least-squares fitting of a line to the initial rise in 5' and 3' mRNA signals and identify intercepts to the basal levels (**Figure 4B**). The difference between these intercepts indicates the average time for RNAPs to travel from the 5' probe region to the 3' probe region. Divide the distance between two probe sets (2 kb) with this time to obtain the average rate of transcription elongation.

9.6.2. To obtain the rate of mRNA degradation, fit an exponential decay function, $y = A \cdot \exp(-t/\tau)$ to the final decay region of the 5' and 3' mRNA signals (e.g., **Figure 4B**). The fitting parameter, $\tau$, is the average mRNA lifetime.

9.7. (Optional) Analyze the cell-to-cell variation in gene expression (e.g., the cell-level response to the induction shown in **Figure 4C**), based on the distribution of mRNA numbers in each cell (calculated in Step 9.5).

9.8. (Optional) Using information about spot location along the major and minor axes of a cell (obtained from Step 9.2), analyze the localization of mRNAs (**Figure 4D**,**E**).

9.9. (Optional) Analyze co-localization of 5' and 3' mRNAs (**Figure 5**) by comparing localization of spots detected in the Cy5 and Cy3 channels.

9.9.1. Load images of multi-color beads (Step 8.6) in the spotFinderF function in microbeTracker and obtain coordinates of bead centroids in Cy5 and Cy3 channels. Use the list of centroid coordinates to calculate the affine transformation matrix, which informs how Cy5 and Cy3 channels are shifted and rotated with respect to each other[29].

9.9.2. Apply the affine transformation matrix to Cy5 and Cy3 FISH images to convert Cy3 images in the Cy5 coordinate. Classify if a spot is co-colocalized with another spot in a different channel. For example, a spot in the Cy5 channel is co-localized with another spot in the Cy3 channel if the distance between their centroids is less than 150 nm (**Figure 5**).

9.9.3. Analyze how many Cy5 spots are classified as "co-localized" with Cy3 spots at each time point. Also, analyze the intensity of the co-localized spots (**Figure 5**). This is done in testColocalization.m file.

**REPRESENTATIVE RESULTS**

**Figure 3** shows representative images from this smFISH protocol. A full field of view (86.7 µm x 66.0 µm using our microscopy setup detailed in **Table of Materials**) shows ~500 *E. coli* cells dispersed throughout the field (**Figure 3A**). If the density of cells is higher than what is shown in this image, automatic cell segmentation becomes difficult as segmentation algorithms do not reliably identify individual cells when cells touch each other. One needs to adjust the

concentration of cells and incubation time used for surface adherence (Step 5.1) to achieve the optimal density of cells in the field of view.

The morphology of cells in the phase contrast images should remain comparable to that of live cells for segmentation purposes (**Figure 3A**-**3C**). If cells are over-permeabilized, the cell morphology changes (like "ghosts"; **Supplementary Figure 1**). In that case, one may reduce the duration of 70% ethanol treatment in Step 5.3.

When the expression level of *lacZ* mRNA is low, there are one or two diffraction-limited *lacZ* mRNA spots spatially separated within a cell. Hence, these spots can be analyzed by 2D Gaussian fitting for their intensity and localization.

In our experimental condition, ~0.6% of cells contain *lacZ* mRNA (detected by either 5' or 3' mRNA probes) before induction, and the average expression level was ~0.03 *lacZ* mRNAs per cell, consistent with previous reports[15,30]. Also, the distribution of *lacZ* mRNA spot intensities before induction did not fit well with a normal distribution or a Poisson distribution due to the presence of spots with high intensities (**Figure 3D**-**3E**). This suggests that most of the spots detected under the repressed state represent a single *lacZ* mRNA, but a small population of spots contains more than one *lacZ* mRNA. To isolate the population with a single *lacZ* mRNA, we used a Gaussian mixture model with two mixture components (insets in **Figure 3D**-**3E**). Then, the mean of the first Gaussian was taken as the mean intensity of a single mRNA spot (e.g., the peak of the black curve in **Figure 3D**) and used to convert the spot intensity to the number of mRNAs, for any spots detected in the time-course experiment. To calculate the total number of mRNAs within a cell, the normalized spot intensities were summed in each cell (**Figure 3F**)[19].

When the expression level is high, such that spots overlap with each other within a cell, 2D Gaussian fitting does not do reliable quantification. In that case, the mRNA level should be calculated by dividing the total, background-subtracted fluorescence signal within a cell with the mean intensity of a single mRNA[19].

When the expression of *lacZ* is induced, the signal of 5' *lacZ* mRNA increases first and that of 3' *lacZ* mRNA increases later (**Figure 4B**). If the expression of *lacZ* is repressed, both 5' and 3' *lacZ* mRNA signals decrease with some delay in between (**Figure 4B**). To obtain the rate of transcription elongation, the rise of 5' and 3' signals are first fit with lines (**Figure 4B**) because the difference in x-intercepts indicates the time RNAPs take to travel the distance between two probe regions (2,000 nt). The rate of transcription elongation can be measured from each time-course experiment and standard deviations can be calculated from experimental duplicates. The average rate of transcription elongation was 15-30 nt/s under our experimental conditions[19].

Additionally, the rate of mRNA degradation (inverse of the mean mRNA lifetime) was obtained by fitting the decay region with an exponential function (**Figure 4B**). Our time-course data contains mRNA degradation during and after transcription[31]. We fit the time points after 3'

mRNA started to decay (t > 6 min) to probe the degradation of released mRNAs. We obtained ~90 s as an average lifetime of either 5' or 3' *lacZ* mRNA[19].

The rate of transcription initiation can be calculated from the slope of 5' signal increase after induction (**Figure 4B**, blue), or from the average mRNA number at steady state (which is the initiation rate divided by the degradation rate). Furthermore, the probability of premature transcription termination can be estimated, either by taking the ratio between the slope of 3' signal increase vs that of 5' signal increase[32] or between the steady-state levels of 3' and 5' mRNA regions[19].

Because smFISH is a single-cell technique, we can analyze cell-to-cell variability in transcription. For example, one can analyze the percentage of cells expressing *lacZ* mRNA after IPTG is added (**Figure 4C**). One can also address whether mRNA localization changes after induction. We observed that 5' and 3' *lacZ* mRNA spots move slightly outward, away from the center of the cell (**Figure 4D,E**), consistent with a previous report[33].

Lastly, analysis of co-localization between 5' and 3' mRNA spots can be informative (**Figure 5A**). For example, in the repressed state (time zero), about 25% of 5' mRNA spots are co-localized with a 3' mRNA spot. At t = 1 min, as many gene loci have 5' mRNA synthesis, but not yet 3' mRNA synthesis, most of the 5' mRNA spots are by themselves without 3' mRNA signal (i.e., low probability of co-localization). However, when the 3' mRNA appears (i.e., t = 2 min), the probability of co-localization increases (purple arrow in **Figure 5A**,**B**). This time point, when the co-localization becomes frequent, depends on the rate of transcription elongation. The 2-D density plot of 5' and 3' *lacZ* mRNA numbers within each co-localization spot, at this time point, suggests the density of RNAPs on the *lacZ* gene (**Figure 5C**). As previously reported[19], the 5' mRNA numbers in this plot indicate that most of the *lacZ* loci have less than 10 RNAPs on the DNA when *lacZ* expression is induced by 1 mM IPTG. Additionally, the 3' mRNA numbers in this plot imply that RNAPs do not form clusters[34]. The fact that the number of 3' mRNA is close to one means that roughly only one RNAP enters the 3' probe region. This suggests that RNAPs on the *lacZ* gene are spatially separated, instead of forming a cluster (or "convoy").

**FIGURE LEGENDS**

**Figure 1: Design of smFISH probes for an mRNA of interest.** (**A**) A tiling method. Sequences of short DNA oligonucleotides (~20 bp in length) are chosen so that they can cover the mRNA of interest. The oligonucleotide probes are labeled with a fluorescent dye molecule. (**B**) An array method. A non-coding array of tandem sequences (e.g., "*lacO* array") is transcriptionally fused to the mRNA of interest. Fluorescently labeled probe complementary to the repeat unit (e.g., lacO probe of 17 bp in length) is used to amplify the signal of an mRNA.

**Figure 2: Schematic of smFISH experimental procedure and time duration of each step.**

**Figure 3: smFISH image analysis.** (**A**-**C**) smFISH microscopy image of 5' *lacZ* mRNA (red) and 3' *lacZ* mRNA (green) in wild-type *E. coli* (MG1655) grown in M9 minimal medium supplemented

with 0.2% glycerol, 0.1% casamino acids, and 1 mg/L thiamine at 30 °C. (**A**) A representative image of a sample from t = 3 min after induction with 0.05 mM IPTG at t = 0 min and repression with 500 mM glucose at t = 1.5 min. Phase contrast and two fluorescence images of Cy5 (for 5' *lacZ* mRNA, red) and Cy3 (for 3' *lacZ* mRNA, green) were overlaid with pseudo-coloring. The image shows an entire field of 86.7 µm x 66.0 µm. Scale bar, 5 µm. (**B**) Zoom-in version of a small region (yellow box) in (A). Cell outlines are shown in white, and fluorescence spots identified from image analysis are shown in red. Scale bar, 1 µm. (**C**) Detection of cell outlines and fluorescent spots under a high expression condition (t = 4 min after induction with 1 mM IPTG). Scale bar, 1 µm. (**D**-**E**) Distributions of 5' and 3' mRNA spot intensities measured before adding IPTG (the repressed state). The histograms are shown with two Gaussian functions (black and grey) whose mean values are from the Gaussian mixture model. Inset shows quantile-quantile plot of random numbers generated from the Gaussian mixture models and experimentally measured mRNA spot intensities (n = 1040 for 5' mRNA and 680 for 3' mRNA). (**F**) Information obtained for an individual cell pointed in panel (B). For a given cell (i), spots were identified in Cy5 and Cy3 channels, and their intensity (I) and coordinate along the short and long axis of a cell (*d*, *l*) were quantified from 2D Gaussian fitting. The spot intensities were used to calculate the total number of 5' or 3' mRNAs in this cell. Also, co-localization between spots from different channels can be analyzed as in the example shown in **Figure 5**.

**Figure 4: Analysis of in vivo kinetics of transcription and mRNA degradation.** (**A**) Schematic and representative images of two-color smFISH experiments measuring changes in *lacZ* mRNA levels over time. Red and green dotted lines indicate Cy5 or Cy3B labeled oligonucleotide probes that hybridize to the 1-kb-long 5' and 3' mRNA regions of *lacZ* mRNA in *E. coli*, respectively. Also shown are overlays of two fluorescence images with a phase contrast image at indicated time points after induction with 0.2 mM IPTG at t = 0 min. Transcription was repressed with 500 mM glucose at t = 1.5 min. Scale bar, 1 µm. The figure has been modified from Kim et al[19]. (**B**) 5' and 3' *lacZ* mRNA numbers per cell over time, during the experiment described in the panel (A). Error bars are bootstrapped SEMs. At least 1,200 cells were analyzed per time point. The initial rise of the 5' and 3' mRNA signals was fit with a line (blue). The difference in x-intercepts was 1.93 min, yielding the average rate of transcription elongation of 17.3 nt/s. Final decay of the 5' and 3' mRNA signals was fit with an exponential decay function (grey). The fit parameters indicate that the average mRNA lifetime is 1.52 min for 5' mRNA and 1.66 min for 3' mRNA. (**C**) Percentage of cells with one or more *lacZ* mRNA spots during the experiment described in (A). Error bars are bootstrapped SEMs. (**D**) Localization of a spot along a cell's short axis. One can quantify a spot's proximity to the membrane by dividing the location along the short axis (*d*) with half width of the cell (*w*). (**E**) Change in the localization of 5' and 3' *lacZ* mRNA spots along cells' short axis during the experiment described in (A).

**Figure 5: Analysis of co-localization of 5' and 3' mRNA spots.** (**A**) Schematic showing the expected co-localization between 5' and 3' mRNA spots after induction. When 3' mRNA is made, the probability of a 5' mRNA spot being co-localized with a 3' mRNA spot increases (purple arrow). (**B**) The probability of co-localization after induction with 1 mM IPTG. The purple arrow indicates the time point where the probability of co-localization first becomes frequent according to the schematic in panel (A). (**C**) The number of 5' and 3' *lacZ* mRNAs within a co-

localization spot detected at t = 2 min after induction with 1 mM IPTG (total 841 spots). Gray dots represent individual co-localized spots, whereas red dots represent the average of binned data. Error bars are SEM. The shade of gray indicates the density of points in a given area of the graph. The dotted line indicates a slope of 1.

**Figure 6: Optimization of the probe hybridization condition.** Two kinds of samples were used: MG1655 cells grown as described in **Figure 3** and remain uninduced (blue) or treated with 0.5 mM IPTG for 20 min (red). Probe hybridization solution was made with different concentrations of probes (total 72 Cy5-conjugated probes tiling the entire *lacZ* region) and of formamide. Formamide concentrations were also adjusted in the pre-hybridization solution and the wash solution, accordingly. "No probe" (grey line) indicates the fluorescence level of the IPTG-added cells treated with no probes during the hybridization step. Mean fluorescence intensity normalized by cell area (AU) was calculated from 300-800 cells. Error bars are bootstrapped SEMs.

**Supplementary Figure 1:** Distorted cell morphologies due to over permeabilization. Overlay of phase contrast (gray scale), 5' lacZ mRNA (Cy5, red), and 3' lacZ mRNA (Cy3, green) images of MG1655 cells 5 min after the induction with 1 mM IPTG. (**A**) An example showing mixture of normall cells and overly permeabilized cells lacking normal morphology (indicated with pink arrows). (**B**) An example showing "ghosty" cells clumped together. Scale bar = 1 μm.

**Table 1: Recipes of the solutions used.**

## DISCUSSION

Here, we presented a smFISH protocol for measuring mRNA kinetics in *E. coli*. In the previously published smFISH protocols for bacteria[23], cells were kept in the tubes until the very end of the protocol, that is until they are ready for imaging. While it has many benefits, such as minimal nonspecific binding of fluorescent probes on the coverslip surface[23], it is difficult to follow these protocols for many samples acquired from a time-course experiment. First, a relatively large volume of cells (>1 mL) needs to be sampled and even harvested before fixation. Second, the cell samples need to be centrifuged multiple times to exchange solutions and to wash after the hybridization step. In our protocol, a small volume (<1 mL) of culture is directly mixed with a fixing solution in a 1.5-mL tube, helping to quickly "freeze" the cell state at the moment of sampling. Also, cells stay attached to the surface throughout the procedure, and different solutions can be exchanged quickly by aspirating liquids with a vacuum filtration system and applying solution drops at once with a multi-channel pipette. This difference makes our protocol highly advantageous when a large number of samples need to be processed at once. Using our protocol, 12-48 samples can be handled simultaneously and the entire FISH procedure can be completed within ~8 hours, about a similar amount of time needed for a few samples (**Figure 2**). Although we used the expression of *lacZ* in *E. coli* as an example, the protocol is widely applicable to different genes and bacterial species with considerations discussed below.

For different genes, the first thing to consider is smFISH probes. One may design oligonucleotide probes that tile the mRNA of interest (**Figure 1A**)[13]. In this "tiling" probe approach, each probe is ~20 base long and labeled with a fluorophore at the 5' or 3' terminus. This strategy is convenient as no genetic manipulation is needed. Alternatively, an array of ~20 bp sequence, foreign to the genomic sequence (e.g., an array of *lacO* sequence in *Caulobacter crescentus*[14]), may be inserted in the untranslated region of a gene of interest and a single probe complementary to the repeat unit is used to label the mRNA ("array" approach; **Figure 1B**). In both cases, multiple fluorophores decorate an mRNA, giving amplified fluorescence signal that can be easily differentiated from a single probe nonspecifically bound inside a cell.

Whether to choose "tiling" or "array" approaches depends on the negative control, a sample where nonspecific binding of probes is tested because it lacks the target mRNA. For tiling probes (**Figure 1A**), a mutant strain without the gene of interest or a condition, in which the gene is not transcribed (e.g., repression of *lacZ*) can serve as a negative control for testing nonspecific binding of probes. For array-based smFISH (**Figure 1B**), a wild-type strain lacking the array can serve as a negative control because it does not contain binding sites for the probes.

Optimal hybridization conditions may depend on the probe sequences and even the choice of fluorophore dyes. We optimized the hybridization condition for *lacZ* probe sets by keeping the hybridization temperature at 37 °C and testing different concentrations of probe sets and formamide in the hybridization solution. Higher concentrations of formamide tend to reduce both nonspecific and specific binding[26,35]. We recommend systematically changing the hybridization and its wash conditions while keeping hybridization time and temperature the same. As the condition becomes more stringent, both nonspecific and specific binding decrease (**Figure 6**). It is important to find a point where the nonspecific binding starts to hit below an acceptable threshold without further compromising specific binding. For example, we used the signal level obtained without any probes ("no probes") as a threshold (**Figure 6**).

The two-color smFISH method labeling two separate regions of an mRNA is limited to long genes. For measuring the rate of transcription elongation, we took advantage of the fact that *lacZ* is long (3075 bp) and its expression can be induced by IPTG. When a gene is short, it is difficult to design two tiling probe sets (near 5' and 3' ends) and resolve the time delay between appearances of 5' vs. 3' mRNA regions. In this case, one may count nascent mRNAs at steady state by smFISH and analyze their distribution with an analytical model that has the rate of transcription elongation as a fitting parameter[20]. Also, when a gene of interest is not inducible, one may treat cells with rifampicin at time zero and measure the temporal change in 5' and 3' mRNA sub-regions. The delay from the decrease of 5' mRNA signal to that of 3' mRNA signal can then be used to calculate the rate of transcription elongation as done previously[31].

Finally, the smFISH protocol is versatile and can be combined with other labeling schemes. Previously, DNA locus was visualized together with mRNAs by combining mRNA FISH with either DNA FISH[14] or fluorescent reporter-operator system[20]. Protein products may also be visualized by performing immunofluorescence together with mRNA FISH[14,36]. Also, it can be combined

with three-dimensional super-resolution microscopy[37] to visualize mRNAs in all three dimensions[38,39].

**ACKNOWLEDGMENTS**

This protocol was developed by S.K. during her postdoctoral research in Dr. Christine Jacobs-Wagner's laboratory at the Howard Hughes Medical Institute and the Microbial Sciences Institute at Yale University. We thank Dr. Jacobs-Wagner and her lab members for various inputs during the method development. S.K. acknowledges support from the Searle Scholars Program; K.V. acknowledges the support of James Scholar Preble Research Award from the University of Illinois.

**DISCLOSURES**

The authors declare they have no competing financial interests.

**REFERENCES**

1 Bervoets, I., Charlier, D. Diversity, versatility and complexity of bacterial gene regulation mechanisms: opportunities and drawbacks for applications in synthetic biology. *FEMS Microbiology Reviews.* **43** (3), 304-339 (2019).

2 Epshtein, V., Nudler, E. Cooperation between RNA polymerase molecules in transcription elongation. *Science.* **300** (5620), 801-805 (2003).

3 Vogel, U., Jensen, K. F. The RNA chain elongation rate in Escherichia coli depends on the growth rate. *Journal of Bacteriology.* **176** (10), 2807-2813 (1994).

4 Tennyson, C. N., Klamut, H. J., Worton, R. G. The human dystrophin gene requires 16 hours to be transcribed and is cotranscriptionally spliced. *Nature Genetics.* **9** 184 (1995).

5 Singh, J., Padgett, R. A. Rates of in situ transcription and splicing in large human genes. *Nature Structural & Molecular Biology.* **16** 1128 (2009).

6 Selinger, D. W., Saxena, R. M., Cheung, K. J., Church, G. M., Rosenow, C. Global RNA Half-Life Analysis in Escherichia coli Reveals Positional Patterns of Transcript Degradation. *Genome Research.* **13** (2), 216-223 (2003).

7 Bernstein, J. A., Khodursky, A. B., Lin, P.-H., Lin-Chao, S., Cohen, S. N. Global analysis of mRNA decay and abundance in Escherichia coli at single-gene resolution using two-color fluorescent DNA microarrays. *Proceedings of the National Academy of Sciences.* **99** (15), 9697-9702 (2002).

8 Pérez-Ortín, J. E., Medina, D. A., Chávez, S., Moreno, J. What do you mean by transcription rate? *BioEssays.* **35** (12), 1056-1062 (2013).

9 Tang, F. et al. mRNA-Seq whole-transcriptome analysis of a single cell. *Nature Methods.* **6** (5), 377-382 (2009).

10 Kuchina, A. et al. Microbial single-cell RNA sequencing by split-pool barcoding. *BioRxiv.* 10.1101/869248 869248 (2019).

11 Blattman, S. B., Jiang, W., Oikonomou, P., Tavazoie, S. Prokaryotic single-cell RNA sequencing by in situ combinatorial indexing. *Nature Microbiology.* s41564-020-0729-6 (2020).

12 Femino, A., Fay, F., Fogarty, K., Singer, R. Visualization of single RNA transcripts in situ. *Science.* **280** (5363), 585-590 (1998).

13 Raj, A., van den Bogaard, P., Rifkin, S. A., van Oudenaarden, A., Tyagi, S. Imaging individual mRNA molecules using multiple singly labeled probes. *Nature Methods.* **5** (10), 877-879 (2008).
14 Montero Llopis, P. et al. Spatial organization of the flow of genetic information in bacteria. *Nature.* **466** (7302), 77-81 (2010).
15 So, L.-h. et al. General properties of transcriptional time series in Escherichia coli. *Nature Genetics.* **43** (6), 554-560 (2011).
16 Taniguchi, Y. et al. Quantifying E. coli proteome and transcriptome with single-molecule sensitivity in single cells. *Science.* **329** (5991), 533-538 (2010).
17 Jones, D. L., Brewster, R. C., Phillips, R. Promoter architecture dictates cell-to-cell variability in gene expression. *Science.* **346** (6216), 1533-1536 (2014).
18 Iyer, S., Park, B. R., Kim, M. Absolute quantitative measurement of transcriptional kinetic parameters in vivo. *Nucleic Acids Research.* **44** (18), e142 (2016).
19 Kim, S., Beltran, B., Irnov, I., Jacobs-Wagner, C. Long-Distance Cooperative and Antagonistic RNA Polymerase Dynamics via DNA Supercoiling. *Cell.* **179** (1), 106-119 (2019).
20 Wang, M., Zhang, J., Xu, H., Golding, I. Measuring transcription at a single gene copy reveals hidden drivers of bacterial individuality. *Nature Microbiology.* **4** (12), 2118-2127 (2019).
21 Joo, C., Ha, T. Labeling DNA (or RNA) for single-molecule FRET. *Cold Spring Harbor Protocols.* **2012** (9), 1005-1008 (2012).
22 Sambrook, J., Russell, D. W. Standard Ethanol Precipitation of DNA in Microcentrifuge Tubes. *Cold Spring Harbor Protocols.* **2006** (1), pdb.prot4456 (2006).
23 Skinner, S. O., Sepúlveda, L. A., Xu, H., Golding, I. Measuring mRNA copy number in individual Escherichia coli cells using single-molecule fluorescent in situ hybridization. *Nature Protocols.* **8** (6), 1100-1113 (2013).
24 Adesnik, M., Levinthal, C. The synthesis and degradation of lactose operon messenger RNA in E. coli. *Cold Spring Harbor Symposia on Quantitative Biology.* **35**, 451-459 (1970).
25 Campbell, E. A. et al. Structural mechanism for rifampicin inhibition of bacterial RNA polymerase. *Cell.* **104** (6), 901-912 (2001).
26 Raj, A., Tyagi, S. in *Methods in Enzymology* Vol. 472 (ed Nils G. Walter) 365-386 (Academic Press, 2010).
27 Sliusarenko, O., Heinritz, J., Emonet, T., Jacobs-Wagner, C. High-throughput, subpixel precision analysis of bacterial morphogenesis and intracellular spatio-temporal dynamics. *Molecular Microbiology.* **80** (3), 612-627 (2011).
28 Paintdakhi, A. et al. Oufti: an integrated software package for high-accuracy, high-throughput quantitative microscopy analysis. *Molecular Microbiology.* **99** (4), 767-777 (2016).
29 Moffitt, J. R., Zhuang, X. in *Methods in Enzymology* Vol. 572 eds Grigory S. Filonov & Samie R. Jaffrey) 1-49 (Academic Press, 2016).
30 Yu, J., Xiao, J., Ren, X., Lao, K., Xie, X. S. Probing gene expression in live cells, one protein molecule at a time. *Science.* **311** (5767), 1600-1603 (2006).
31 Chen, H., Shiroguchi, K., Ge, H., Xie, X. S. Genome-wide study of mRNA degradation and transcript elongation in Escherichia coli. *Molecular Systems Biology.* **11** (1), 781 (2015).
32 Vogel, U., Sørensen, M., Pedersen, S., Jensen, K. F., Kilstrup, M. Decreasing transcription elongation rate in Escherichia Coli exposed to amino acid starvation. *Molecular Microbiology.* **6** (15), 2191-2200 (1992).

33 Yang, S. et al. Transcription and translation contribute to gene locus relocation to the nucleoid periphery in E. coli. *Nature Communications.* **10** (1), 5131 (2019).
34 Zenklusen, D., Larson, D. R., Singer, R. H. Single-RNA counting reveals alternative modes of gene expression in yeast. *Nature Structural & Molecular Biology.* **15** (12), 1263-1271 (2008).
35 Fontenete, S., Guimarães, N., Wengel, J., Azevedo, N. F. Prediction of melting temperatures in fluorescence in situ hybridization (FISH) procedures using thermodynamic models. *Critical Reviews in Biotechnology.* **36** (3), 566-577 (2016).
36 Sepúlveda, L. A., Xu, H., Zhang, J., Wang, M., Golding, I. Measurement of gene regulation in individual cells reveals rapid switching between promoter states. *Science.* **351** (6278), 1218-1222 (2016).
37 Huang, B., Wang, W., Bates, M., Zhuang, X. Three-Dimensional Super-Resolution Imaging by Stochastic Optical Reconstruction Microscopy. *Science.* **319** (5864), 810-813 (2008).
38 Moffitt, J. R., Pandey, S., Boettiger, A. N., Wang, S., Zhuang, X. Spatial organization shapes the turnover of a bacterial transcriptome. *eLife.* **5** e13065 (2016).
39 Fei, J. et al. Determination of in vivo target search kinetics of regulatory noncoding RNA. *Science.* **347** (6228), 1371-1374 (2015).

Figure 1



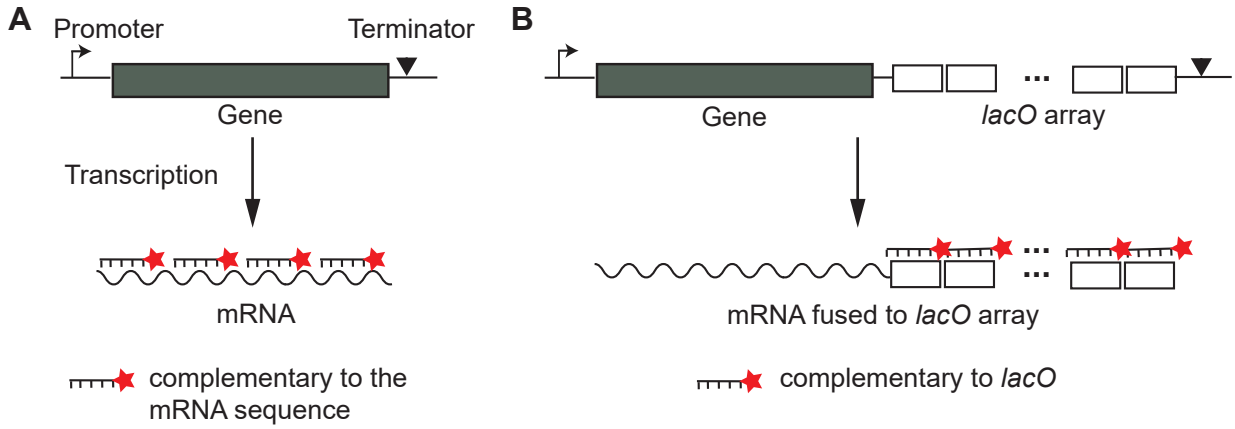



Figure 1

Figure 2



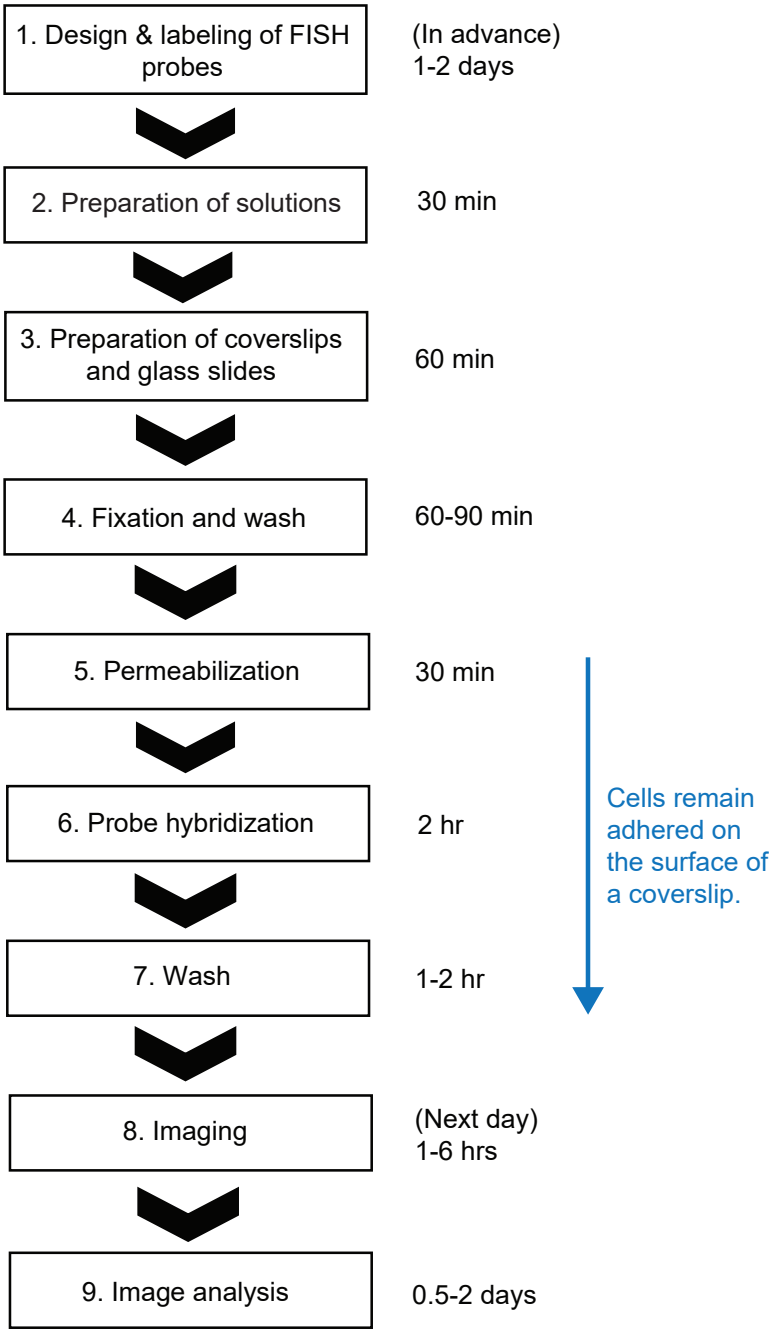



Figure 2

Figure 3



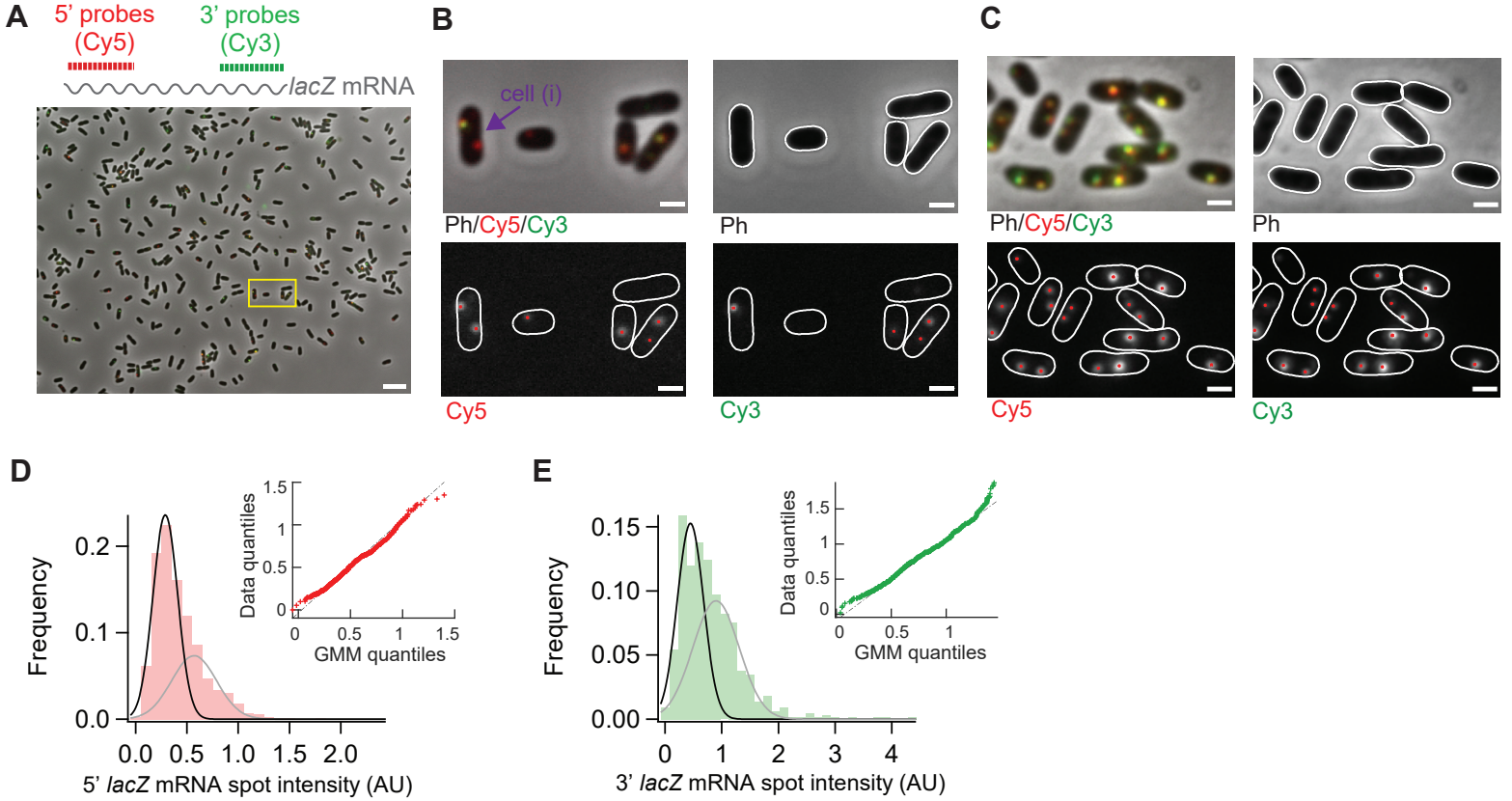



Figure 3

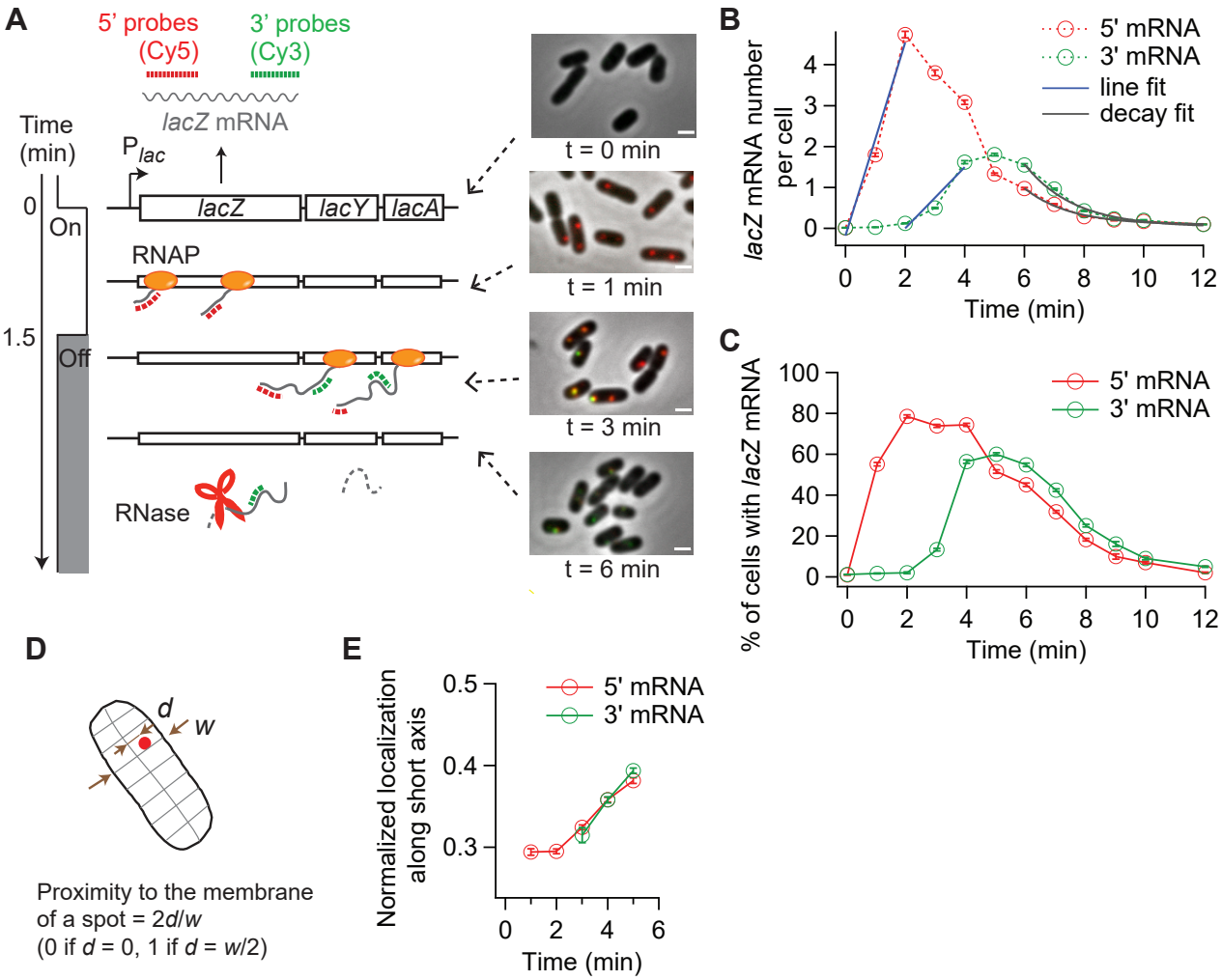



Figure 4

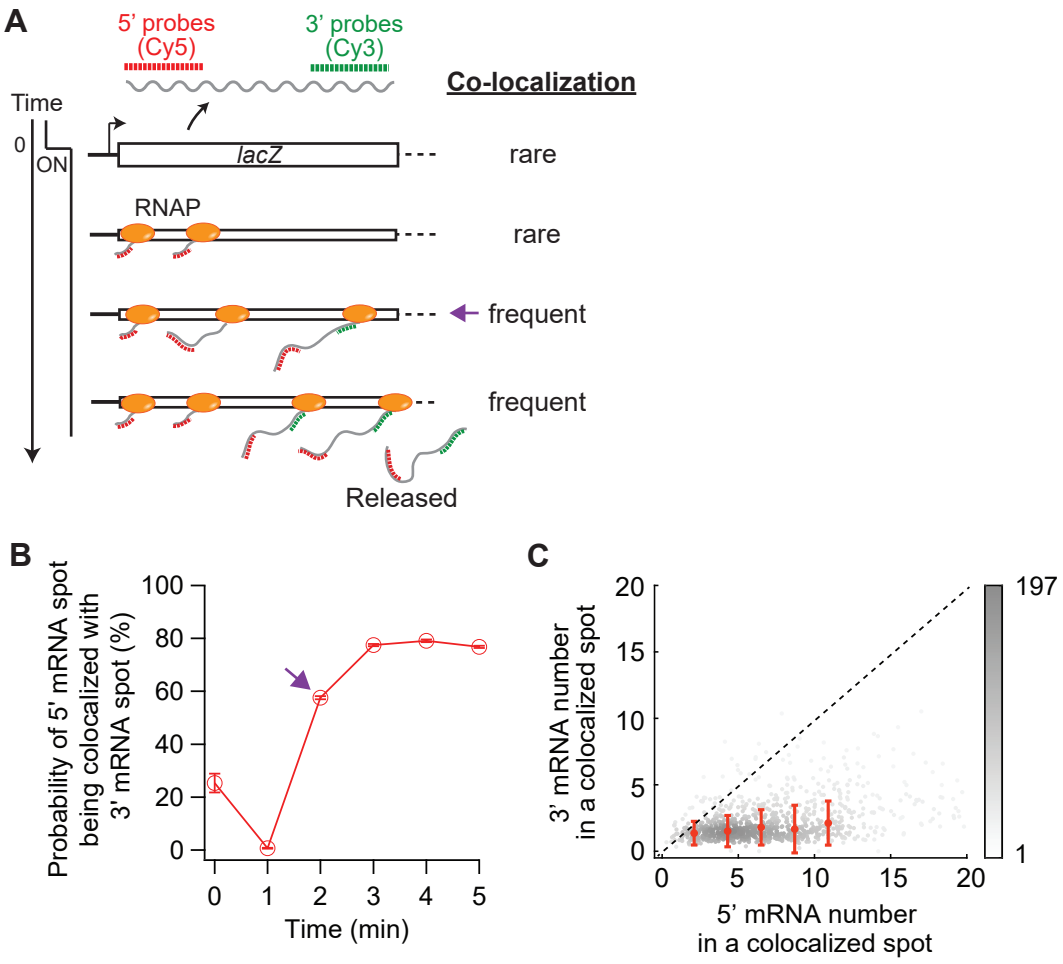



Figure 5

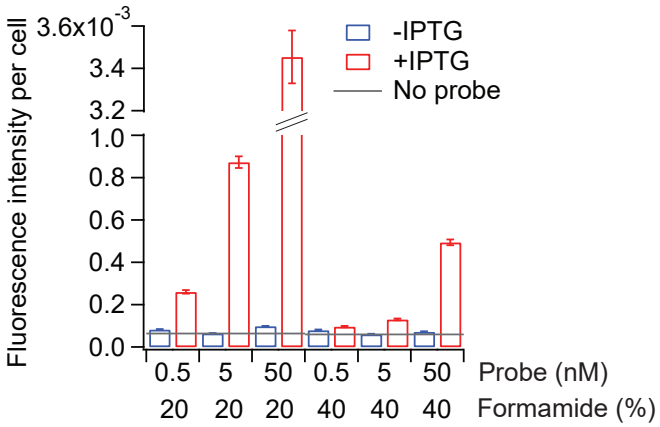



Figure 6

**DEPC Water**

Add 0.1% DEPC to ultrapure water and incubate the bottle (covered) in the 37°C oven overnight and autoclave next day.

**DEPC PBS (10X)**

Mix the following:

| | |
|---|---|
| 80 g | NaCl (final 1.37 M) |
| 2 g | KCl (final 27 mM) |
| 14.2 g | $Na_2HPO_4$ (final 100 mM) |
| 2.7 g | $KH_2PO_4$ (final 20 mM) |

Ultrapure water to 1L

Filter (0.22 µm) into a glass bottle.

Add 0.1% DEPC and follow the instruction for DEPC water.

To make 1X solution, dilute 10 times with DEPC water.

**1M DEPC sodium phosphate buffer, pH 7.4**

Mix the following:

| | |
|---|---|
| 115 g | $Na_2HPO_4$ |
| 22.8 g | $NaH_2PO_4$ |

Ultrapure water to 1 L

Filter (0.22 µm) into a glass bottle.

Add 0.1% DEPC and follow the instruction for DEPC water.

**4X fixing solution (16% formaldehyde)**

| | |
|---|---|
| 5 mL | 20% formaldehyde |
| 500 µL | DEPC water |
| 750 µL | 1M DEPC sodium phosphate buffer, pH 7.4 |

Store at 4 °C for up to 2-4 weeks.

CAUTION: Formaldehyde is toxic. Wear gloves and use a fume hood when making this solution.

**Wash solution**

Mix the following:

| | |
|---|---|
| 10 mL | Formamide (final 25%) |
| 4 mL | 20X SSC (final 2X) |

Fill DEPC water to 40 mL

Filter (0.22 µm) and store at 4 °C

CAUTION: Formamide is toxic. Wear gloves and use a fume hood when making this solution.

**Pre-hybridization solution**

| | |
|---|---|
| 200 µL | Formamide (final 20%) |
| 100 µL | 20X SSC (final 2X) |
| 10 µL | 100X VRC (final 1X) |
| 25 µL | 4% (w/v) BSA (final 0.1%) |
| 685 µL | DEPC water |

NOTE: Vortex the VRC stock before taking 10 µL out.

CAUTION: Formamide is toxic and a known teratogen. Wear gloves and handle it under a fume hood.

**Probe hybridization solution**

| | |
|---|---|
| 200 µL | Formamide (final 20%) |
| 100 µL | 20X SSC (final 2X) |
| 10 µL | 100X VRC (final 1X) |
| 25 µL | 4% (w/v) BSA (final 0.1%) |
| 10 µL | 40 mg/mL *E. coli* tRNA (final 0.4 mg/mL) |
| 200 µL | 50% dextran sulfate (final 10%) |
| x µL | 5’ mRNA probe set (from Step 1.12) to final 4 nM. |
| y µL | 3’ mRNA probe set (from Step 1.12) to final 4 nM. |
| - | DEPC water to make the total volume 1 mL |

NOTE: Add dextran sulfate last. Because it is very viscous, cut the end of a pipette tip before taking 200 µL out from the 50% stock. After adding dextran sulfate, pipette up and down to homogenize the solution.

| Name of Material/Equipment | Company | Catalog Number |
|---|---|---|
| Bacterial strain | | |
| *Escherichia coli* MG1655 | | |
| | | |
| Chemicals, peptides, and others | | |
| Acetonitrile | Sigma-Aldrich | 34851 |
| Ammonium chloride | Fisher Chemical | A661-500 |
| Bovine serum albumin (BSA) | Sigma-Aldrich | B2518 |
| Calcium chloride | Acros Organics | 349610250 |
| Casamino acid | BD Difco | 223050 |
| Cy3B NHS ester | GE Healthcare Life Sciences | PA63101 |
| Cy5 NHS ester | GE Healthcare Life Sciences | PA15101 |
| DEPC | Sigma-Aldrich | D5758 |
| Dextran sulfate | Millipore | S4030 |
| Dextrose | Fisher Chemical | D16 |
| Dimethyl sulfoxide (DMSO) | Sigma-Aldrich | D8418 |
| *E. coli* tRNA | Sigma-Aldrich | R1753 |
| Ethanol | Decon Laboratories | 2701 |
| FISH probes | Biosearch Technologies | |
| Formaldehyde | Ladd Research Industries | 20295 |
| Formamide | American Bio | AB00600 |
| Glycerol | Americanbio | AB00751-01000 |
| Isopropylthio-β-galactoside (IPTG) | Invitrogen | 15529019 |
| Magnesium sulfate | Fisher Chemical | M65-500 |
| 2-Nitrophenyl β-D-fucopyranoside (ON | Santa Cruz Biotechnology | sc-216258 |
| Picodent twinsil 22 | Picodent | 1300 1000 |
| Poly-L-lysine | Sigma-Aldrich | P8920 |
| Potassium chloride | Fisher BioReagents | BP366-500 |
| Potassium phosphate monobasic | Fisher BioReagents | BP362-500 |
| Rifampicin | Sigma-Aldrich | R3501 |
| Saline-sodium citrate buffer (SSC) | Invitrogen | AM9763 |
| sodium bicarbonate | Fisher BioReagents | BP328-500 |
| Sodium chloride | Fisher BioReagents | BP358-1 |
| Sodium phosphate dibasic | Fisher BioReagents | BP332-500 |

| | | |
|---|---|---|
| Sodium phosphate monobasic | Fisher BioReagents | BP329-500 |
| Super PAP Pen | Invitrogen | 8899 |
| TetraSpek microspheres | Invitrogen | T7280 |
| Thiamine | Sigma-Aldrich | T1270 |
| Triethylammonium acetate | Sigma-Aldrich | 90358 |
| Vanadyl ribonucleoside complex (VRC | Sigma-Aldrich | 94742 |

| Equipment | | |
|---|---|---|
| C18 column | Waters | Acquity BEH C18 column |
| Countertop centrifuge | Eppendorf | 5425 |
| Countertop incubator | Eppendorf | Thermomixer F1.5 |
| Incubator (Oven) | Thermo Scientific | 51030514 |
| Water purification system | Millipore | Milli-Q Reference |
| Nanodrop | Thermo Scientific | 2000C |
| Nitrogen gas | Building | |
| UPLC | Waters | Acquity UPLC system |
| Vacuum and aspirator | Building | |
| Vacuum concentrator | Labconco | 7810010 |
| Vortexer | Scientific Industries | Genie-2 SI-0236 |
| Water bath shaker | New Brunswick | Innova 3100 |
| Water bath sonicator | VWR | 97043-960 |

| Tools | | |
|---|---|---|
| 1.5-mL tubes | Eppendorf | 22431021 |
| 1000-uL pipette tip box | Denville Scientific | P1126 |
| Coplin jar | SPI | 01240-AB |
| Coverslip | Fisher Scientific | 22-050-230 |
| Filtered pipette tips | Denville Scientific | P1121,P1122,P1126 |
| Forceps | SPI | K35a |
| Glass slide | Fisher Scientific | 12-544-1 |
| Gloves | Microflex | MK-296-M |
| Multichannel pipetter | Eppendorf | 2231300045 |
| Pipette | Gilson | P1000, P200, P20 |
| Reagent reservoir | MTC Bio | P8025-1S |

| | | |
|---|---|---|
| Syringe filter (0.22 um) | Millipore | SLGS033SS |
| Timer | VWR | 62344-641 |
| **Software and algorithms** | | |
| MATLAB | Mathworks | R2013 and up |
| MicrobeTracker or Oufti | | |
| Stellaris Probe Designer | Biosearch Technologies | |
| **Microscope** | | |
| CCD camera | Hamamatsu Photonics | Orca-II-ER |
| Cy3 filter set | Chroma | 49004 |
| Cy5 filter set | Chroma | 49006 |
| Epi-fluorescence microscope | Nikon | Eclipse Ti |
| Fluorescence excitation source | Lumencor | SOLA-E |
| Nikon Elements software | Nikon | |
| Phase-contrast 100x objective | Nikon | Plan Apochromat (NA 1.45) |
| **Probe sequence** | | |
| DNA oligos with C6 amino modification at the 5' end | Biosearch Technologies Inc | |
| lacZ1 | | GTGAATCCGTAATCATGGTC |
| lacZ2 | | TCACGACGTTGTAAAACGAC |
| lacZ3 | | ATTAAGTTGGGTAACGCCAG |
| lacZ4 | | TATTACGCCAGCTGGCGAAA |
| lacZ5 | | ATTCAGGCTGCGCAACTGTT |
| lacZ6 | | AAACCAGGCAAAGCGCCATT |
| lacZ7 | | AGTATCGGCCTCAGGAAGAT |
| lacZ8 | | AACCGTGCATCTGCCAGTTT |
| lacZ9 | | TAGGTCACGTTGGTGTAGAT |
| lacZ10 | | AATGTGAGCGAGTAACAACC |
| lacZ11 | | GTAGCCAGCTTTCATCAACA |
| lacZ12 | | AATAATTCGCGTCTGGCCTT |
| lacZ13 | | AGATGAAACGCCGAGTTAAC |

| | |
|---|---|
| lacZ14 | AATTCAGACGGCAAACGACT |
| lacZ15 | TTTCTCCGGCGCGTAAAAAT |
| lacZ16 | ATCTTCCAGATAACTGCCGT |
| lacZ17 | AACGAGACGTCACGGAAAAT |
| lacZ18 | GCTGATTTGTGTAGTCGGTT |
| lacZ19 | TTAAAGCGAGTGGCAACATG |
| lacZ20 | AACTGTTACCCGTAGGTAGT |
| lacZ21 | ATAATTTCACCGCCGAAAGG |
| lacZ22 | TTTCGACGTTCAGACGTAGT |
| lacZ23 | ATAGAGATTCGGGATTTCGG |
| lacZ24 | TTCTGCTTCAATCAGCGTGC |
| lacZ25 | ACCATTTTCAATCCGCACCT |
| lacZ26 | TTAACGCCTCGAATCAGCAA |
| lacZ27 | ATGCAGAGGATGATGCTCGT |
| lacZ28 | TCTGCTCATCCATGACCTGA |
| lacZ29 | TTCATCAGCAGGATATCCTG |
| lacZ30 | CACGGCGTTAAAGTTGTTCT |
| lacZ31 | TGGTTCGGATAATGCGAACA |
| lacZ32 | TTCATCCACCACATACAGGC |
| lacZ33 | TGCCGTGGGTTTCAATATTG |
| lacZ34 | ATCGGTCAGACGATTCATTG |
| lacZ35 | TGATCACACTCGGGTGATTA |
| lacZ36 | ATACAGCGCGTCGTGATTAG |
| lacZ37 | GATCGACAGATTTGATCCAG |
| lacZ38 | AAATAATATCGGTGGCCGTG |
| lacZ39 | TTTGATGGACCATTTCGGCA |
| lacZ40 | TATTCGCAAAGGATCAGCGG |
| lacZ41 | AAGACTGTTACCCATCGCGT |
| lacZ42 | TGCCAGTATTTAGCGAAACC |
| lacZ43 | AAACGGGGATACTGACGAAA |
| lacZ44 | TAATCAGCGACTGATCCACC |
| lacZ45 | GGGTTGCCGTTTTCATCATA |
| lacZ46 | TCGGCGTATCGCCAAAATCA |
| lacZ47 | TTCATACAGAACTGGCGATC |

| | |
|---|---|
| lacZ48 | TGGTGTTTTGCTTCCGTCAG |
| lacZ49 | ACGGAACTGGAAAAACTGCT |
| lacZ50 | TATTCGCTGGTCACTTCGAT |
| lacZ51 | GTTATCGCTATGACGGAACA |
| lacZ52 | TTTACCTTGTGGAGCGACAT |
| lacZ53 | GTTCAGGCAGTTCAATCAAC |
| lacZ54 | TTGCACTACGCGTACTGTGA |
| lacZ55 | AGCGTCACACTGAGGTTTTC |
| lacZ56 | ATTTCGCTGGTGGTCAGATG |
| lacZ57 | ACCCAGCTCGATGCAAAAAT |
| lacZ58 | CGGTTAAATTGCCAACGCTT |
| lacZ59 | CTGTGAAAGAAAGCCTGACT |
| lacZ60 | GGCGTCAGCAGTTGTTTTTT |
| lacZ61 | TACGCCAATGTCGTTATCCA |
| lacZ62 | TAAGGTTTTCCCCTGATGCT |
| lacZ63 | ATCAATCCGGTAGGTTTTCC |
| lacZ64 | GTAATCGCCATTTGACCACT |
| lacZ65 | AGTTTTCTTGCGGCCCTAAT |
| lacZ66 | ATGTCTGACAATGGCAGATC |
| lacZ67 | ATAATTCAATTCGCGCGTCC |
| lacZ68 | TGATGTTGAACTGGAAGTCG |
| lacZ69 | TCAGTTGCTGTTGACTGTAG |
| lacZ70 | ATTCAGCCATGTGCCTTCTT |
| lacZ71 | AATCCCCATATGGAAACCGT |
| lacZ72 | AGACCAACTGGTAATGGTAG |

**Comments/Description**

UPLC buffer B
To make M9 medium
Probe hybridization
To make M9 medium
To make M9 medium
Fluorophore for FISH probes
Fluorophore for FISH probes

Probe hybridization
To repress the expression of lacZ
To dissolve fluorophores
Probe hybridization
Used in DNA purification, lysis, and cleaning coverslips
Sequences are published in ref#16
Fixation
Probe hybridization, pre-hybridization, and wash
To make M9 medium
*lacZ* induction
To make M9 medium
*lacZ* repression
Sealant
To treat the coverslip surface
To make PBS
To make PBS and M9 medium
To stop transcription initiation
Probe hybridization, pre-hybridization, and wash
Fluorophore-probe conjugation
For DNA purification, PBS and M9 medium
To make PBS and M9 medium

To make a sodium phosphate buffer
Hydrophobic marker for coverslips
Controls for multi-channel registration
To make M9 medium
UPLC buffer A
Probe hybridization and pre-hybridization

Gravity convection

For blow-drying coverslips and glass slides

Aspirator is made of a filtration flask with a side arm.
Centrivap; to dry samples collected from UPLC.

Critical for time-course experiments
To clean coverslips and glass slides

DNA lobind tubes
An empty box after using all the tips
To clean coverslips and glass slides
24x60 No1
SHARP® Precision Barrier Tips
To handle clean coverslips and glass slides

To use in the washing step (#7)

To use in the washing step (#7)

https://www.mathworks.com
https://www.github.com/JacobsWagnerLab/MicrobeTracker
https://oufti.org/
https://www.biosearchtech.com/support/tools/design-software/stellaris-probe-designer

For phase-contrast and epi fluorescence

software that controls the microscope setup

5' mRNA
5' mRNA
5' mRNA
5' mRNA
5' mRNA
5' mRNA
5' mRNA
5' mRNA
5' mRNA
5' mRNA
5' mRNA
5' mRNA
5' mRNA
5' mRNA

5' mRNA
5' mRNA
5' mRNA
5' mRNA
5' mRNA
5' mRNA
5' mRNA
5' mRNA
5' mRNA
5' mRNA
5' mRNA

3' mRNA
3' mRNA
3' mRNA
3' mRNA
3' mRNA
3' mRNA
3' mRNA
3' mRNA
3' mRNA
3' mRNA
3' mRNA
3' mRNA
3' mRNA
3' mRNA
3' mRNA
3' mRNA
3' mRNA
3' mRNA
3' mRNA
3' mRNA
3' mRNA
3' mRNA
3' mRNA
3' mRNA
3' mRNA

**Editorial comments:**

**Please see our answers written in blue.**

1. The editor has formatted the manuscript to match the journal style. Please retain and use the attached version for revision.

Thank you very much for handling our manuscript. We kept the changed format and made textual changes only.

2. Please address all the specific comments marked in the manuscript.

We addressed all the comments marked in the document file. In particular, we made changes in the procedure section to make the steps concise.

3. Once done please ensure that the highlight is no more than 2.75 pages.

Our highlight is under 2.75 pages.

# ELSEVIER LICENSE TERMS AND CONDITIONS

May 19, 2020

This Agreement between Sangjin Kim ("You") and Elsevier ("Elsevier") consists of your license details and the terms and conditions provided by Elsevier and Copyright Clearance Center.

| | |
|---|---|
| License Number | 4830091252155 |
| License date | May 15, 2020 |
| Licensed Content Publisher | Elsevier |
| Licensed Content Publication | Cell |
| Licensed Content Title | Long-Distance Cooperative and Antagonistic RNA Polymerase Dynamics via DNA Supercoiling |
| Licensed Content Author | Sangjin Kim,Bruno Beltran,Irnov Irnov,Christine Jacobs-Wagner |
| Licensed Content Date | Sep 19, 2019 |
| Licensed Content Volume | 179 |
| Licensed Content Issue | 1 |
| Licensed Content Pages | 30 |
| Start Page | 106 |
| End Page | 119.e16 |
| Type of Use | reuse in a journal/magazine |
| Requestor type | academic/educational institute |
| Portion | figures/tables/illustrations |
| Number of figures/tables/illustrations | 1 |
| Format | electronic |
| Are you the author of this Elsevier article? | Yes |
| Will you be translating? | No |
| Title of new article | Probing mRNA kinetics in space and time in Escherichia coli using two-color single-molecule fluorescence in situ hybridization |
| Lead author | Sangjin Kim |
| Title of targeted journal | Journal of Visualized Experiments |
| Publisher | MyJove Corp. |
| Expected publication date | Nov 2020 |
| Portions | Figure 1D |
| Requestor Location | Sangjin Kim<br>104 S Goodwin Ave Rm 190N<br><br>CHAMPAIGN, IL 61822<br>United States<br>Attn: Sangjin Kim |
| Publisher Tax ID | 98-0397604 |
| Total | **0.00 USD** |
| Terms and Conditions | |

**INTRODUCTION**

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions

established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

**GENERAL TERMS**

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.
3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:
"Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."
4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.
5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com). No modifications can be made to any Lancet figures/tables and they must be reproduced in full.
6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.
7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.
8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.
9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.
10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.
11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.
12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).
13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.
14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

**LIMITED LICENSE**

The following terms and conditions apply only to specific license types:
15. **Translation**: This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article.
16. **Posting licensed content on any Website**: The following terms and conditions apply as follows: Licensing material from an Elsevier journal: All content posted to the web site must maintain the copyright information line on the bottom of each image; A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx or the Elsevier homepage for books at http://www.elsevier.com; Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

Licensing material from an Elsevier book: A hyper-text link must be included to the Elsevier homepage at http://www.elsevier.com . All content posted to the web site must maintain the copyright information line on the bottom of each image.

**Posting licensed content on Electronic reserve**: In addition to the above the following clauses are applicable: The web site must be password-protected and made available only to bona fide students registered on a relevant course. This permission is granted for 1 year only. You may obtain a new license for future website posting.

17. **For journal authors:** the following clauses are applicable in addition to the above:

**Preprints:**

A preprint is an author's own write-up of research results and analysis, it has not been peer-reviewed, nor has it had any other value added to it by a publisher (such as formatting, copyright, technical enhancement etc.).

Authors can share their preprints anywhere at any time. Preprints should not be added to or enhanced in any way in order to appear more like, or to substitute for, the final versions of articles however authors can update their preprints on arXiv or RePEc with their Accepted Author Manuscript (see below).

If accepted for publication, we encourage authors to link from the preprint to their formal publication via its DOI. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help users to find, access, cite and use the best available version. Please note that Cell Press, The Lancet and some society-owned have different preprint policies. Information on these policies is available on the journal homepage.

**Accepted Author Manuscripts:** An accepted author manuscript is the manuscript of an article that has been accepted for publication and which typically includes author-incorporated changes suggested during submission, peer review and editor-author communications.

Authors can share their accepted author manuscript:

- immediately
  - via their non-commercial person homepage or blog
  - by updating a preprint in arXiv or RePEc with the accepted manuscript
  - via their research institute or institutional repository for internal institutional uses or as part of an invitation-only research collaboration work-group
  - directly by providing copies to their students or to research collaborators for their personal use
  - for private scholarly sharing as part of an invitation-only work group on commercial sites with which Elsevier has an agreement
- After the embargo period
  - via non-commercial hosting platforms such as their institutional repository
  - via commercial sites with which Elsevier has an agreement

In all cases accepted manuscripts should:

- link to the formal publication via its DOI
- bear a CC-BY-NC-ND license - this is easy to do
- if aggregated with other manuscripts, for example in a repository or other site, be shared in alignment with our hosting policy not be added to or enhanced in any way to appear more like, or to substitute for, the published journal article.

**Published journal article (JPA):** A published journal article (PJA) is the definitive final record of published research that appears or will appear in the journal and embodies all value-adding publishing activities including peer review co-ordination, copy-editing, formatting, (if relevant) pagination and online enrichment.

Policies for sharing publishing journal articles differ for subscription and gold open access articles:

**Subscription Articles:** If you are an author, please share a link to your article rather than the full-text. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help your users to find, access, cite, and use the best available version.

Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

If you are affiliated with a library that subscribes to ScienceDirect you have additional private sharing rights for others' research accessed under that agreement. This includes use for classroom teaching and internal training at the institution (including use in course packs and courseware programs), and inclusion of the article for grant funding purposes.

**Gold Open Access Articles:** May be shared according to the author-selected end-user license and should contain a CrossMark logo, the end user license, and a DOI link to the formal publication on ScienceDirect.

Please refer to Elsevier's posting policy for further information.

18. **For book authors** the following clauses are applicable in addition to the above: Authors are permitted to place a brief summary of their work online only. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version. **Posting to a repository:** Authors are permitted to post a summary of their chapter only in their institution's repository.

19. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include

permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for Proquest/UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission. Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

**Elsevier Open Access Terms and Conditions**

You can publish open access with Elsevier in hundreds of open access journals or in nearly 2000 established subscription journals that support open access publishing. Permitted third party re-use of these open access articles is defined by the author's choice of Creative Commons user license. See our open access license policy for more information.

**Terms & Conditions applicable to all Open Access articles published with Elsevier:**

Any reuse of the article must not represent the author as endorsing the adaptation of the article nor should the article be modified in such a way as to damage the author's honour or reputation. If any changes have been made, such changes must be clearly indicated.

The author(s) must be appropriately credited and we ask that you include the end user license and a DOI link to the formal publication on ScienceDirect.

If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source it is the responsibility of the user to ensure their reuse complies with the terms and conditions determined by the rights holder.

**Additional Terms & Conditions applicable to each Creative Commons user license:**

**CC BY:** The CC-BY license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article and to make commercial use of the Article (including reuse and/or resale of the Article by commercial entities), provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by/4.0.

**CC BY NC SA:** The CC BY-NC-SA license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article, provided this is not done for commercial purposes, and that the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. Further, any new works must be made available on the same conditions. The full details of the license are available at http://creativecommons.org/licenses/by-nc-sa/4.0.

**CC BY NC ND:** The CC BY-NC-ND license allows users to copy and distribute the Article, provided this is not done for commercial purposes and further does not permit distribution of the Article if it is changed or edited in any way, and provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, and that the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by-nc-nd/4.0. Any commercial reuse of Open Access articles published with a CC BY NC SA or CC BY NC ND license requires permission from Elsevier and will be subject to a fee.

Commercial reuse includes:

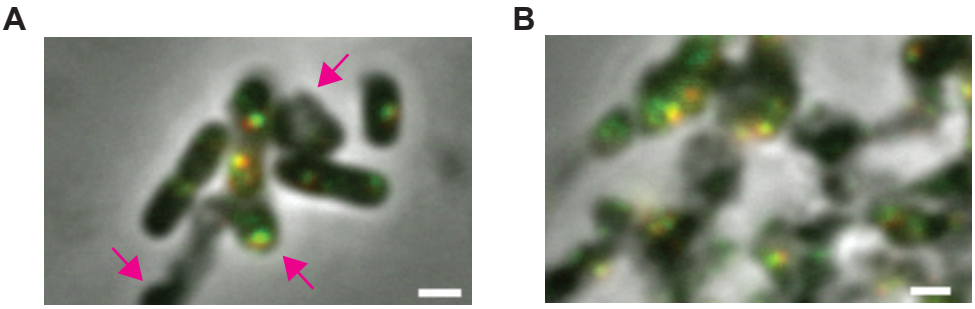
- Associating advertising with the full text of the Article
- Charging fees for document delivery or access
- Article aggregation
- Systematic distribution via e-mail lists or share buttons

Posting or linking by commercial companies for use by customers of those companies.

20. **Other Conditions**:

v1.9

**Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.**




# Supplementary Figure 1

Distorted cell morphologies due to over permeabilization. Overlay of phase contrast (gray scale), 5' lacZ mRNA (Cy5, red), and 3' lacZ mRNA (Cy3, green) images of MG1655 cells 5 min after the induction with 1 mM IPTG. (A) An example showing mixture of normall cells and overly permeabilized cells lacking normal morphology (indicated with pink arrows). (B) An example showing "ghosty" cells clumped together. Scale bar = 1 μm.