Chapter 9

Single-Molecule Fluorescence In Situ Hybridization (smFISH) for RNA Localization Relative to a DNA Locus in Bacteria

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Abstract

When studying the localization of RNA within a cell, it is important to probe RNA relative to a subcellular landmark, such as a gene locus (transcription site). In this chapter, we describe a single-molecule fluorescence in situ hybridization (smFISH) protocol for labeling target RNAs and their DNA loci in bacteria. This is a versatile protocol applicable to various genes and species, providing valuable insights into RNA localization in bacterial cells.

Keywords smFISH, DNA FISH, FROS, DNA loci, Bacteria, RNA localization

1 Introduction

Understanding the cellular mechanisms that regulate RNA metabolism requires a method for precise measurement of RNA copy number and their spatial distribution within a cell. Fluorescence in situ hybridization (FISH) is a powerful technique for these measurements in RNA research [1]. FISH is based on the hybridization of fluorescently labeled single-stranded oligonucleotides to the DNAs and RNAs of interest, enabling the visualization of specific nucleic acid sequences at their native locations within cells [2]. Here, we will use the term FISH to refer to the labeling of target RNA while DNA FISH refers to the labeling of target DNA [3]. Despite the limitation of FISH methods to fixed cells, they exhibit superior target specificity based on the sequence complementarity. Also, FISH can detect a single RNA molecule (single-molecule FISH or smFISH) [4, 5] suitable for measuring both low-copy and high-copy RNA species

Sangjin Kim designed the original protocol. Juan Echeverry detailed the protocol and generated scripts. Yu-Huan Wang optimized the analysis protocol. All authors read and approved the final version of the manuscript.

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[6–8]. FISH can be combined with super-resolution microscopy for higher spatial resolution beyond the diffraction limit of light [9, 10]. There are high-throughput implementations of FISH that allow the visualization of many gene transcripts from the same cells [11–14]. Different transcripts can be visualized simultaneously by using probes of spectrally distinct fluorophores [15] or successively from multiple rounds of hybridization and washing steps [11, 12]. Subregions of an mRNA can be distinguished by using spectrally distinct fluorophores for their probes to show the presence or absence of the subregions due to transcription elongation and mRNA degradation [16–19] or splicing in eukaryotic cells [20]. Unlike traditional bulk RNA analysis methods (e.g., Northern blot [21] or real-time PCR [22]), FISH is a single-cell method, providing cell-to-cell variations within a population.

In recent years, FISH has been widely used to study gene expression kinetics [16–19] and subcellular localization of RNAs in bacteria [23–28]. In a model bacterium, *Escherichia coli*, many mRNAs are expressed to an average copy number of less than 1 [7]. Therefore, the single-molecule sensitivity of FISH is critical for accurately counting the number of RNAs. Furthermore, the FISH technique has allowed researchers to view the complex organization of the bacterial transcriptome in cells. Based on the diffusion of RNA in the cytoplasm, RNAs were expected to be present at random locations within a cell [23, 29]. However, a study showed that several mRNAs in *Caulobacter crescentus* and *lacZ* mRNA in *E. coli* localize near the site of transcription [23]. Another study reported that *E. coli* mRNAs localize to where their encoded proteins localize for their cellular functions [24]. Later, a high-throughput super-resolution FISH study in *E. coli* showed that mRNAs encoding inner-membrane proteins localize near the membrane while other mRNAs are distributed in the cytoplasm [14, 27]. The extensive variations observed in mRNA localization have attracted considerable attention from the scientific community [30]. In eukaryotic cells, mRNA localization in distinct membrane-bound organelles is a well-established mechanism for spatially restricting protein synthesis [31]. However, in bacteria, the mechanisms and functional significance of mRNA localization are still being elucidated.

Considering the life cycle of an RNA from birth (transcription) to death (mRNA degradation), RNA can be present at the gene locus during transcription, before releasing for free diffusion (Fig. 1a). In FISH images, RNAs being transcribed (nascent RNAs) should exhibit colocalization with the DNA (Fig. 1a). In contrast, released RNAs are more likely to localize away from the gene locus [17] unless there are other mechanisms in play to keep the released transcripts near the locus [17, 23]. Marking a gene locus during FISH enables distinguishing nascent and released RNAs and measuring how far certain RNAs are from their site of transcription.

Previously, we published a smFISH protocol focusing on counting mRNAs in bacteria based on tiling probes and an array probe (Fig. 1b, c) [19]. In this chapter, we describe a FISH procedure to study

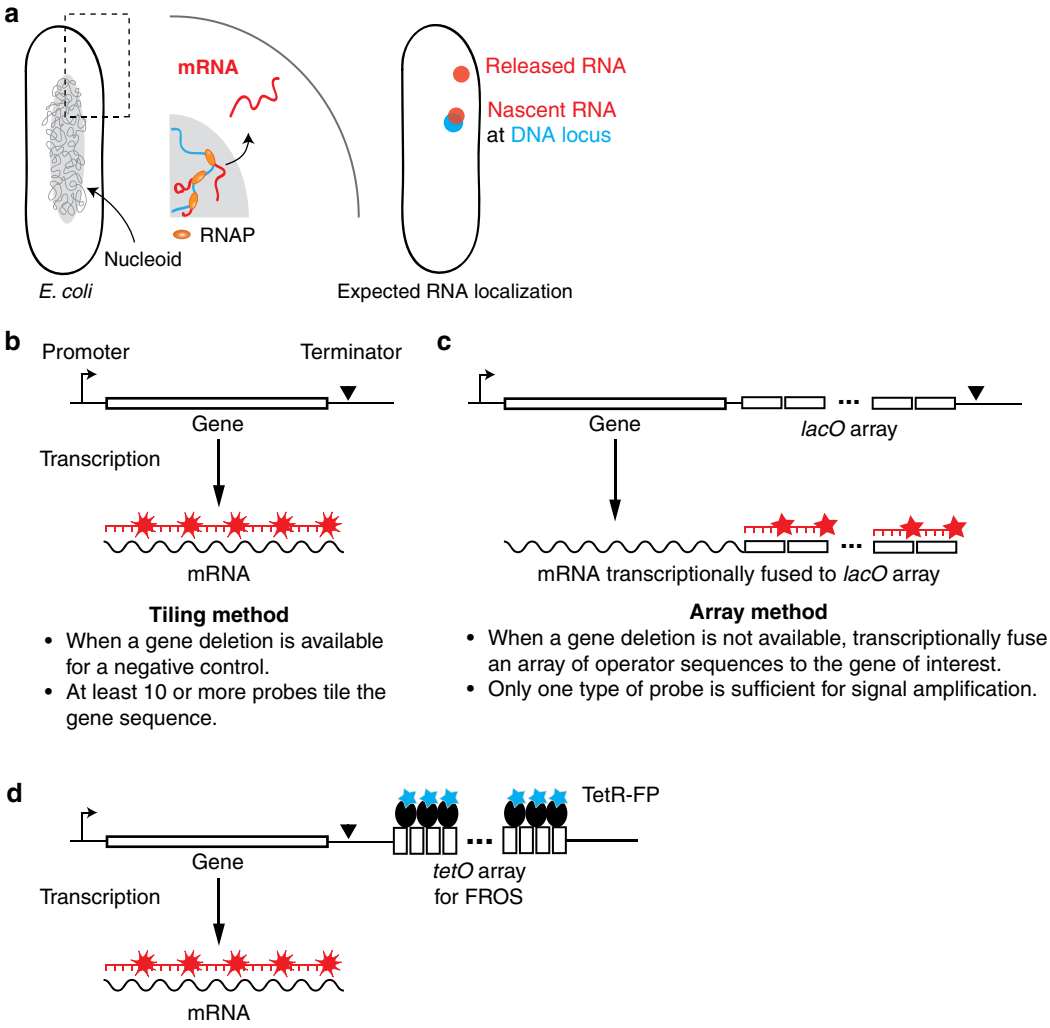

Fig. 1 Overview of FISH in bacteria. (**a**) Expected RNA localization relative to gene loci in bacterial cells. (**b**) and (**c**) Two different probe designs for smFISH in bacteria. (**d**) Gene loci labeling by FROS together with RNA FISH

mRNA localization relative to the transcription site. Chromosomal DNA loci can be visualized either by DNA FISH or fluorescence repressor-operator system (FROS), and the FISH procedure is modified accordingly to accommodate DNA labeling steps.

To perform DNA FISH together with smFISH, an additional step of heating the sample at 95°C is required to denature the DNA double helix at the beginning of the probe hybridization step [32]. DNA FISH probes may target the gene of interest itself or the region outside the gene via the tiling method (like RNA FISH shown in Fig. 1b). DNA FISH can also be done to an array of repeated sequences (Fig. 1c). In all cases, the probes should be designed to hybridize to the template strand of the DNA, so that they do not label transcripts.

Also, any potential antisense transcription should be checked to avoid labeling antisense transcripts. It is important to confirm that the DNA FISH signal is from DNA, not from any transcripts, by treating the sample with DNase and RNase after the last washing step. True DNA FISH signal will disappear upon the DNase treatment but remain unaffected upon the RNase treatment.

Although DNA FISH has been successfully paired with RNA FISH in bacteria [23], we prefer using the FROS method because of the guaranteed specificity and fewer steps to optimize and troubleshoot. FROS is based on fluorescently labeled repressor proteins bound to an array of its binding sites on the DNA (Fig. 1d) [33, 34]. Unlike DNA FISH, FROS requires genome engineering to insert the repressor binding sites close to the gene of interest and to express the fluorescently labeled repressor proteins. However, the labeling efficiency (% of loci visualized) is much higher than DNA FISH, as long as careful attention is made to minimize the loss of fluorescence during the fixation [35], hybridization, and washing steps. Example images are shown in Fig. 2.

A gene can exist in multiple copies (spatially separated) within a bacterial cell due to chromosome replication [36]. If not all loci are labeled, it can create a false negative for the colocalization analysis of RNA and the DNA locus. Therefore, it is critical to have a high efficiency of DNA labeling. Here, we use fluorescently labeled TetR and *tetO* array as an example of FROS for the chromosomal *lacZ* loci in *E. coli* (Figs. 1d and 2a). The same protocol has been applied to LacI-based FROS in

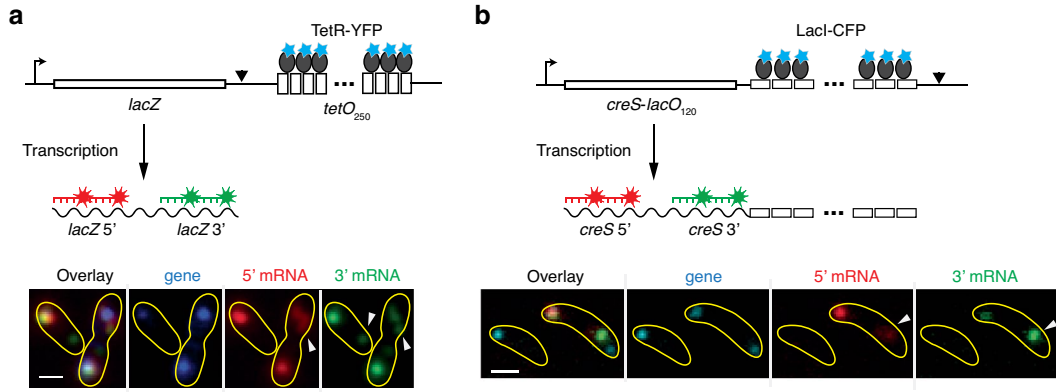

Fig. 2 Example images of FROS and FISH. (**a**) Visualization of *lacZ* mRNA and chromosome loci in *E. coli*. The 5' and 3' mRNA subregions were hybridized with 24 tiling probes labeled with Cy5 and Cy3B, respectively. DNA loci were marked with TetR-YFP bound to a *tetO* array. TetR-YFP was expressed from a pBAD plasmid. 0.02% arabinose was used for induction for 15 min, and cells were washed into fresh media to remove arabinose. *lacZ* mRNA was induced with 0.05 mM IPTG for 15 min before fixation. (**b**) Visualization of *creS* mRNA and chromosome loci in *C. crescentus*. The 5' and 3' mRNA subregions were hybridized with 18 tiling probes labeled with Cy5 and Cy3B, respectively. DNA loci were marked with LacI-CFP bound to a *lacO* array. LacI-CFP was expressed from the xylose locus on the chromosome and induced with 0.03% xylose for 1 h before fixation. In both cases, the majority of the mRNA signal co-localized with FROS (gene loci), with a few mRNA spots found outside the gene loci (white arrows). Scale bar = 1 μm

C. crescentus (Fig. 2b). We will explain strain engineering for FROS and a FISH procedure, which minimizes the loss of FROS signal at the end. Lastly, we will explain ways to analyze data for spatial localization within the cell coordinates. We will also comment on modifications needed for different species and genes in the Notes section.

2 Materials

2.1 Bacterial Cell Culture

1. A strain of interest with FROS capability (*see* **Note 1**).
2. A negative control strain for testing nonspecific binding of probes (*see* **Note 2**).
3. Growth media.

2.2 Fluorescent Probes

1. Unlabeled DNA oligonucleotides with a C6 amino modification at the 5' end.
2. Cy3B NHS ester and Cy5 NHS ester.
3. Cold ethanol.
4. 0.1 M sodium bicarbonate (pH 8.5). Prepare fresh right before usage.
5. 3 M sodium chloride.
6. RNase-free TE buffer: 10 mM Tris-HCl pH 8.0 with 1 mM EDTA.

2.3 smFISH

1. DEPC water: MilliQ water with 0.1% diethyl pyrocarbonate (DEPC). Mix DEPC with water and incubate the bottle (covered) at 37°C overnight and autoclave it the next day.
2. DEPC PBS: Dissolve 80 g NaCl, 2 g KCl, 14.2 g Na_2HPO_4, and 2.7 g NaH_2PO_4 in 1 L MilliQ water. Filter (0.22 μm) into a glass bottle and add 0.1% DEPC. Follow the process shown for the DEPC water. This is 10× stock, which should be diluted to 1×.
3. 4× formaldehyde fix: mix 5 ml of 20% formaldehyde with 0.75 ml of 1 M DEPC sodium phosphate buffer and 0.5 ml of DEPC water. Store at 4°C.
4. 70% ethanol.
5. Wash solution: 25% formamide and 2× saline-sodium citrate (SSC).
6. Prehybridization solution: 20% formamide, 2× SSC, 2 mM Vanadyl ribonucleoside complexes (VRC), 0.1% Bovine serum albumin (BSA).
7. Probe hybridization solution: 20% formamide, 2× SSC, 2 mM VRC, 0.1% BSA, 0.4 mg/ml *E. coli* tRNA, 10% dextran sulfate, fluorescent probes (*see* **Notes 3** and **4**).
8. Picodent silicone dental gum.

2.4 Coverslip and Glass Slide Preparation

1. Coverslips: 24 × 60 No. 1 or 22 × 50 No. 1.5.
2. Glass slides: 25.4 mm × 76.2 mm × 1 mm.
3. Ethanol.
4. MilliQ water.
5. 0.1% poly-l-lysine.
6. Hydrophobic marker.

2.5 Equipment

1. UV–vis spectrophotometer.
2. Refrigerated centrifuge.
3. Counter-top heat block.
4. Incubated shaker.
5. Incubator or oven for 30°C.
6. Vacuum aspirator.
7. Nitrogen gas.
8. Forceps.
9. Coplin jars.
10. Ultrasonic cleaner.
11. Pipettes and multichannel pipettes.
12. Reagent reservoir.
13. Empty pipette tip box.

2.6 Imaging and Analysis

1. Nikon Eclipse Ti-2 Epi-fluorescence microscope.
2. Nikon Phase-contrast 100× objective (Plan Apochromat NA 1.45).
3. SOLA-E fluorescence excitation source from Lumencor.
4. Hamamatsu ORCA II-ER CCD camera.
5. Filter sets for YFP, Cy3, and Cy5.
6. Nikon Elements software.
7. MATLAB.
8. Oufti (compiled version) [37].
9. U-Track (version 2.3) [38].

3 Methods

To avoid RNA degradation due to environmental RNase, wear gloves and wipe out the bench surface and apparatus (e.g., pipettes) with 70% ethanol before experiments. Use RNase-free plastic consumables and filtered pipette tips. Lastly, prepare solutions using DEPC water as much as possible.

3.1 Construction of a FROS Strain (Before FISH Experiments)

1. Amplify a *tetO* array with an antibiotic cassette by PCR from a template strain or a plasmid. The PCR primers should have 50 bp overhang, targeting the chromosome insertion site.
2. Integrate the *tetO* array into the chromosomal locus of interest via lambda red recombination method based on pKD46 plasmid [39].
3. Remove the antibiotic cassette by the Flp recombination based on pCP20 plasmid [40].
4. Transform the plasmid expressing TetR fused to a fluorescent protein (FP). To adjust the expression level of TetR-FP, use an inducible promoter. To reduce the unbound TetR-FP, which creates a high background signal, use a low-copy plasmid or insert the gene into the chromosome. Also, the FP should be chosen to be spectrally separated from organic dyes used for the FISH probes. We have used YFP or CFP for FROS together with Cy3 and Cy5 for FISH (Fig. 2).
5. Image the live cells and confirm that FROS signal is bright and cellular background is low. If the cellular background is high, TetR-FP expression should be lowered. To determine the cellular background, image a strain without any FP to judge the autofluorescence level under the same imaging condition.
6. Analyze the images and determine the number of FROS foci per cell. The distribution of the number of FROS foci in live cells serves as a benchmark for judging the loss of FROS signal over FISH procedure. Hence, imaging should be carefully performed using the same cell culture to be used for FISH.

3.2 Labeling of Probes (Before FISH Experiments)

1. Design probe sequences using Stellaris Probe Designer online tool in the Biosearchtech website.
2. Perform ethanol precipitation of DNA oligos to remove any impurities.
3. Dissolve the DNA oligos in MilliQ water to a final concentration of 1 mM.
4. Dissolve ~1 mg of dye in DMSO to a final concentration of 20 mM.
5. Mix 25 μl of freshly prepared 0.1 M sodium bicarbonate, 5 μl of 1 mM DNA, and 5 μl of dye stock in an Eppendorf tube. Cover the tube with aluminum foil to avoid exposure to light.
6. Incubate the mixture for 6 h at room temperature with gentle mixing.
7. Add 87.5 μl of cold 100% ethanol and 3.5 μl of 3 M sodium chloride to the mixture. Store the tube at −80°C for at least 30 min to overnight.
8. Centrifuge at 21 000 × *g* for 30 min at 4°C.

9. Remove the supernatant carefully using a pipette. Add cold ethanol and spin the tube again at 21 000 × *g* for 3 min at 4°C. Repeat this washing step three times and remove most of the ethanol by pipette.
10. Leave the tube open to dry the DNA pellet in the air. Ethanol will evaporate. Make sure to block light during this step.
11. Dissolve the pellet in DEPC water or DEPC TE buffer.
12. Check the labeling efficiency by comparing the absorption of the DNA (260 nm) and the conjugated dye. When the absorption is converted to molar concentration, the ratio of DNA to dye should be 1, indicating 100% labeling efficiency. If the efficiency is lower than 0.9, repeat the labeling reaction with a new dye stock. If the efficiency is higher than 1, repeat the ethanol precipitation. In either case, one can run additional purification such as denaturing polyacrylamide gel electrophoresis to separate the labeled and unlabeled DNA as well [41].

3.3 Preparation of the Bacterial Cell Cultures

1. Decide the strains to use for FISH. We always include a strain for negative control, which does not contain target sequences for the FISH probes (e.g., a gene deletion strain).
2. Inoculate colonies in a liquid medium of choice and grow overnight in a temperature-controlled shaker (220 rpm). Make sure to include appropriate antibiotics in case the strain includes plasmids.
3. Dilute the overnight culture with the fresh media of the same type at least 10^4 fold. Achieve the high dilution ratio by a serial dilution of 1/1000 and 1/100. Final volume can be 3 ml (in a culture tube) or 10–20 ml (in a flask), depending on the volume needed for FISH. Appropriate antibiotics should be included.
4. Shake the culture until the optical density at 600 nm becomes 0.2 for early exponential phase. During the growth, an inducer might be added to induce TetR-FP or to induce the gene of interest. Check the duration of induction.

3.4 Coverslip and Glass Slide Preparation (on the Day of FISH)

1. Use forceps to place coverslips and slides in a Coplin jar. Make sure that each coverslip and slide is separated from one another (*see* **Note 5**).
2. Fill the Coplin jar with 100% ethanol.
3. Place the jar in an ultrasonic cleaner and sonicate for 15–20 min.
4. Remove the ethanol into a waste container and rinse the jar three to four times with running MilliQ water.
5. Leave a little water in the jar and fill the rest with 100% ethanol (final ethanol concentration ~70%).
6. Repeat sonification (Step 3) and rinsing (Step 4).

7. Fill the jar with MilliQ water and perform sonication (Step 3).
8. Grab one cover slip or slide with forceps at a time. Apply nitrogen gas to the surface to remove any liquid. Store dried coverslips and slides in a clean covered plastic box, such as a pipette tip box. Slides will not be used until the end of the FISH protocol.
9. For dried coverslips, use a hydrophobic pen to draw circles on the glass surface. These circles serve as wells, in which individual samples will be applied (Fig. 3a). Wait 5–10 min for the ink to dry. This step should be done in a pipette tip box for 1 ml pipettes. If circles are drawn based on the circles in the plastic insert in the box, one can use a multichannel pipette to apply solutions to all wells simultaneously as the distance between wells matches the distance between tips in the multichannel pipette (Fig. 3b).

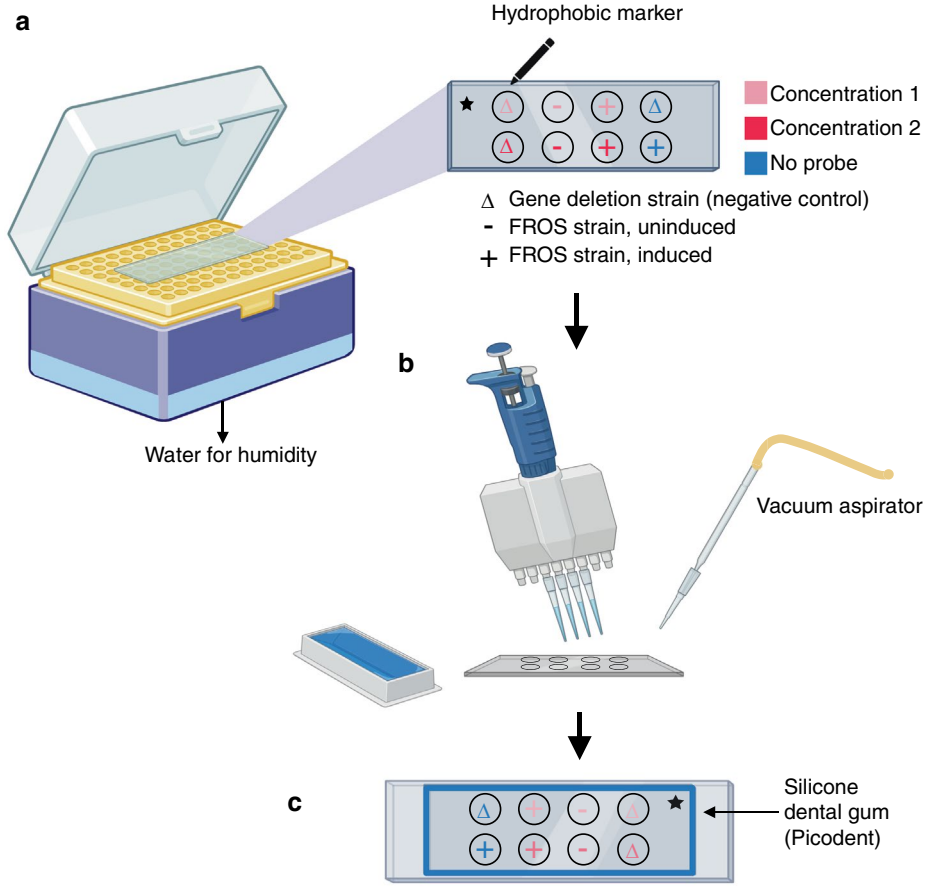

Fig. 3 Schematics of overall experimental procedure for smFISH. (**a**) Arrangement of wells on a cleaned coverslip. Wells are drawn with a hydrophobic marker. (**b**) Solution exchanges with a multi-channel pipette and a vacuum aspirator. (**c**) Mounting of the coverslip onto a cleaned glass slide and sealing with silicon dental gum

10. Apply 20 μl 0.1% poly-L-lysine to each well. If this volume does not cover the well area, apply a larger volume.
11. Wait 10 minutes and then aspirate the poly-L-lysine using a vacuum (*see* **Note 6**). A longer duration is also fine and does not affect the result.
12. Apply 20 μl DEPC water to each well.
13. Store in a closed environment to prevent evaporation.
14. Set aside until ready for use (*see* **Note 5**).

3.5 Cell Fixation and Washing

1. Prepare clean Eppendorf tubes with 300 μl of 4× fixatives.
2. Take 900 μl of cell culture and add it to the fixatives immediately. We do this right next to the shaker. There is no need to vortex or vigorously shake the tube.
3. Incubate at room temperature for 15 min and on ice for 30 min.
4. Centrifuge the tubes at 6500 g for 4 min at 4°C. The speed should be adjusted for different bacterial species (*see* **Note 7**). The cold temperature is critical to avoid fluorescence loss of TetR-FP.
5. Remove the supernatant from the tubes using a pipette and discard it into a chemical waste container (contains formaldehyde).
6. Add 1 ml of cold DEPC PBS to each tube and gently mix with a pipette.
7. Repeat the washing step two more times. Add 100 μl of cold DEPC PBS after the last spin.

3.6 Cell Adhesion

1. Grab the coverslip from Step 14 of Sect. 3.4. Aspirate liquid from wells and add 35 μl of fixed cells to their corresponding wells.
2. Wait 10–30 minutes for cells to adhere to the surface. The time should be adjusted depending on the concentration of cells from Step 7 of Sect. 3.5 (*see* **Note 8**).
3. Aspirate cells and remaining liquid using a vacuum.
4. Apply 35 μl of DEPC PBS to each well.

3.7 Permeabilization of the Cell Membranes

1. Aspirate the liquid and apply 15 μl of 70% cold ethanol. The ethanol will spread on the surface of each well, but not beyond the hydrophobic marker. Start the timer for 4 min. The container can remain uncovered during this step.
2. After 3 min have passed (1 min before the alarm), start to aspirate the ethanol from each well and finish aspirating before the alarm goes off. Be as quick as possible to avoid ethanol contact for more than 4 min. Also, leave the wells exposed to the air for one extra minute to completely dry the surface (*see* **Note 9**).
3. Add 35 μl of the wash solution to each well.

3.8 Prehybridization and Preparation of Hybridization Solution

1. Aspirate the liquid and apply 35 μl of the prehybridization solution to each well.
2. Cover the container and incubate at 30°C for 30 min. To provide humidity, add some water to the bottom of the container (Fig. 3a).
3. While waiting, prepare the hybridization solution and incubate it in a 37°C heat block until ready. Dextran sulfate is very viscous and requires a large orifice pipette tip (simply cut the end using a clean razor blade). Make sure the components are well mixed by gently pipetting up and down but avoid bubble formation. We also prepare no-probe control, which is simply the hybridization solution without any probes. For solutions with fluorescent probes, try to avoid light as much as possible.

3.9 Hybridization, Washing, and Mounting

1. Aspirate the prehybridization solution and add 25 μl of hybridization solution to each well.
2. Cover the container with aluminum foil and incubate at 30°C for 2 h (*see* **Note 10**).
3. Aspirate hybridization solutions. And apply the wash solution to each well.
4. Repeat aspiration and application of new wash solutions four times.
5. With the wash solution in each well, incubate the samples at 30°C for 15 min to help release nonspecifically bound probes to the wash solution.
6. Repeat the above Steps 4 and 5.
7. Repeat Step 4 only.
8. After removing the wash solution, apply 35 μl of DEPC PBS to each well.
9. Aspirate and apply 35 μl of DEPC PBS. Repeat this four times.
10. Leave 35 μl of DEPC PBS in each well until ready.
11. Have a cleaned glass slide ready from Step 8 in Sect. 3.4.
12. Aspirate PBS from each well and apply 3 μl of DEPC PBS to each well.
13. Grab the coverslip from a corner using forceps. Flip it upside down. Hold the coverslip at a 45-degree angle and gently place one edge onto the prepared slide. Slowly lower the other edge of the slide, allowing the PBS to spread and any air to escape.
14. Seal the edge of the coverslip with Picodent (Fig. 3c, *see* **Note 11**).
15. Store at 4°C until ready to image. We typically image the next 1–2 days.

3.10 Imaging

1. Turn on the widefield epifluorescence microscope and prepare imaging with filter sets needed for FROS and FISH labels. Example images are shown in Fig. 2.
2. Drop immersion oil in the middle of the sample and flip to mount on the inverted microscope. The star mark placed at the top left corner of the coverslip should now be oriented at the top left corner as well (Fig. 3a, c). Make sure the sample is flat on the stage. If the silicon seal prevents the sample from lying flat, use a razor blade to cut it out.
3. Raise the objective and find the focus in phase contrast.
4. Use the live mode of phase contrast imaging to look around each well.
5. Take images of regions with many cells. Manually raster scan in each well and try to avoid taking images from an adjacent area.
6. Try to take five to ten images for each well.
7. Save the images to a folder. The Nikon software saves files as nd2 format. Later, these files should be exported into TIFF images for each channel. Phase contrast and fluorescence images will be in separate TIFF files.
8. After imaging is done, the slide can be stored at −20°C.

3.11 Data Analysis

1. Sort image files. Phase contrast images from one well should be in one folder, and corresponding fluorescence images should be in another folder. Different fluorescence channels should be separated into folders (channel 1, channel 2, etc.). This process is done by a MATLAB script called FISHfilePrep.m.
2. Analyze cell meshes using Oufti (Fig. 4a):
   a. Run the compiled version of Oufti as Administrator. This version does not require MATLAB.
   b. Uncheck “stack” since we are loading individual TIFF images.
   c. Load phase contrast TIFF images by selecting the folder containing data of one well.
   d. Select “Independent frames” since we are looking at independent images instead of a time-lapse image.
   e. Select “Load parameters” to load the parameter file for cell detection analysis.
   f. Select “File” to set up the output file for cell detection analysis (test1.mat).
   g. Press “All frames” to run Oufti cell detection. The analysis details will show up in the command window.
   h. After the analysis is done, manually check the result and delete the erroneous detections.

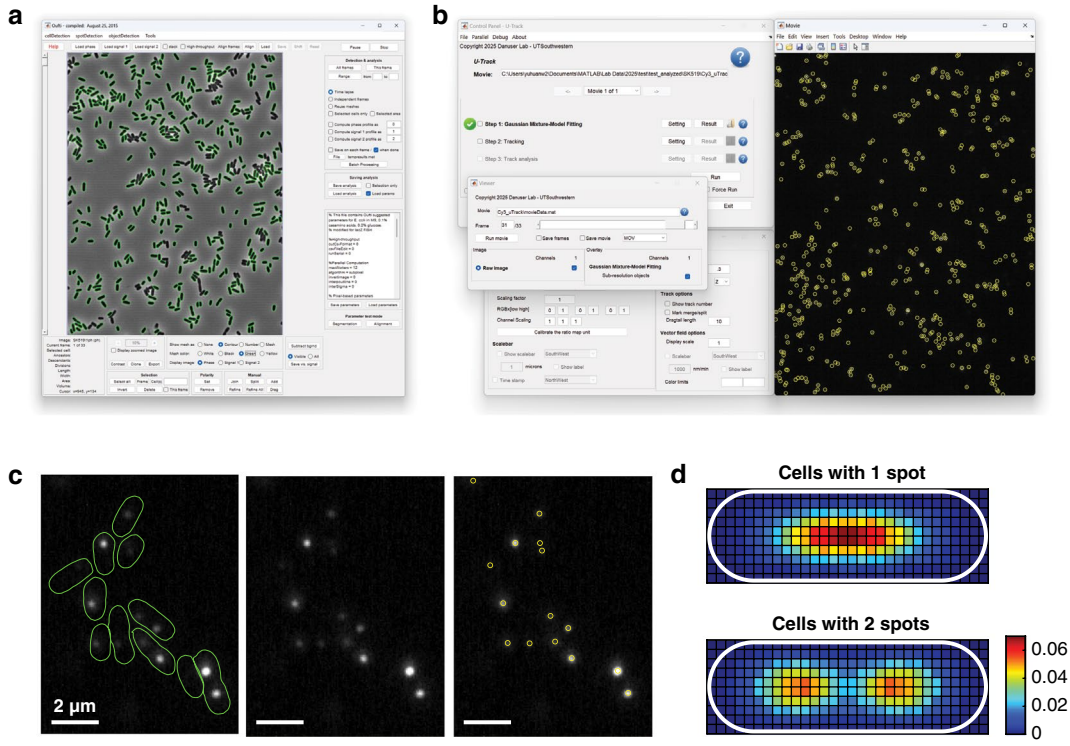

Fig. 4 Data analysis workflow and analysis result. (**a**) Cell detection from Oufti. (**b**) Spot detection from u-track. (**c**) A close-up view of a FISH image with analysis results. Left: with cell outlines from Oufti (green lines). Right: with spots identified by u-track (yellow circles). (**d**) 2D histogram (density heatmap) of spot localization in normalized cellular coordinates. The color represents the probability of finding a spot within a certain 2D bin location. The normalized localization was calculated in the first quartile and extended to the other three quartiles via mirror symmetry. The cell's short and long axes were divided into 10 and 30 bins, respectively. Cells with a single spot, likely due to having one gene copy (*top*), were separately analyzed from cells with two spots, possibly due to having two gene copies (*bottom*)

i. Rename the output file as "-mesh.mat" and select "Reuse meshes" and click "All Frames" to save the finalized result with parameters.

3. Identify spots in fluorescence images using u-track (Fig. 4b). This step requires MATLAB.

   a. Download "u-track" (version 2.3) from the GitHub site of DanuserLab/u-track and add the "u-track" folder and its subfolders into the MATLAB path.

   b. Change the directory to the folder that stores TIFF images.

   c. To launch u-track, enter "movieSelectorGUI" in the MATLAB command window. A GUI window "Movie Selection" will appear.

d. Press “New” to load the FISH images. A GUI window “Movie edition” will appear.

e. Set the input folder as the folder with fluorescent TIFF images (from Step 1) and set the output folder as the folder to save the analysis result.

f. Input your microscope information in the “Movie information” section (leave “Time Interval” as blank since the images are not from a time-lapse).

g. Click “Save” and save the “movieData.mat” in the output folder.

h. After loading the images, press “Continue” to proceed to the analysis part.

i. Select “2D Single Particles” as the object that you want to track. A GUI window “Control Panel – U-Track” will appear.

j. Click the “Setting” for “Step 1: Detection” to set up the spot detection parameters.

k. Choose the default “Gaussian Mixture-Model Fitting” as the detection method and click “Setting.”

l. Input the following parameters (the parameters may need to be optimized for specific experiments).

   - Gaussian Standard Deviation: 1.28 pixels.
   - Alpha-value for Comparison with Local Background: 0.015 (The smaller the alpha value, the stricter the detection).

m. Click “Apply” to save the setup. The checkbox in front of “Step 1” will be automatically checked.

n. Click “Run” to launch the spot detection analysis. MATLAB command window is updated in real time while the program is running.

o. After the analysis is completed, click “Result” to check the analysis result.

p. Check the “Sub-resolution objects” to show the detected spots in each image. Optimize the detection parameters in Step 1 and repeat the analysis.

q. Click “Exit” to exit u-track.

r. Run the script “FISHdataAnalysis.m” to combine Oufti and u-track analysis results and perform further analysis, such as the subcellular position of spots (Fig. 4c, d and *see* **Note 12**).

4 Notes

1. Various FROS systems are available. In addition to TetR/*tetO*, LacR/*lacO* [33, 42] and MalI/*malO* [43] have been used to label gene loci in bacteria. For these, the intensity of the gene loci label is determined by the number of operator binding sites as well as the expression level of the repressor protein. Alternatively, ParB/*parS* requires a small insertion (~300 bp), but many ParB proteins can bind around the *parS* site [44, 45]. High fluorescence spot intensity can be achieved by increasing the expression level of ParB-FP. For all these methods, it is critical to have a high signal-to-background ratio in live cell imaging because the ratio may decrease during the FISH procedure. Excess free repressor proteins (excess compared to the number of binding sites) in the cytoplasm can create a high background in the FROS channel. Therefore, one needs to carefully adjust the expression level of FPs.
2. A negative control sample is from a strain lacking probe targets. In case the gene of interest is non-essential, its deletion mutant can be used [40]. If the gene is essential, one may knock down the gene expression by CRISPRi [46].
3. Prehybridization, hybridization and wash conditions can be optimized for each gene and probe sequences. We test 20–40% formamide and 5–50 nM probe concentrations (see Fig. 3a) to find an optimal condition based on the least nonspecific binding in the negative control and the highest specific signal in the sample.
4. It is important to homogeneously mix the probes in the hybridization solution. Because the hybridization solution is viscous (due to dextran sulfate), one can employ up-down pipetting without creating bubbles to mix the solution (no vortex) and heating the hybridization solution in a 37–42°C heat block beforehand.
5. We recommend cleaning coverslips and glass slides on the day of the experiment. If they are cleaned and stored in water for too long (a few days), the surface becomes hydrophilic, and few cells remain adhered to the surface. Also, solutions spread over the boundary of wells created by the hydrophobic marker, creating cross-talk between wells.
6. Our protocol heavily relies on the vacuum aspirator to remove liquid from the coverslip surface. When the aspirator (glass pipette) is used, we make sure to use fresh pipette tips at the end and try to avoid any contamination between wells. We also keep the tip of the aspirator slightly above the coverslip

surface, primarily along the edge of each well, to avoid touching the center.

7. Different bacterial species may require different centrifugation speeds (Sect. 3.5). For example, 6500 × *g* is used for *E. coli*, and 4500 × *g* is used for *C. crescentus*.
8. It is important to have a good cell density in the well surface. If cells were lost during the washing step (Sect. 3.5), or if the surface adhesion was not long enough (Sect. 3.6), there can be fewer cells in the well, lowering the throughput. We recommend increasing the initial culture volume for fixation and concentrating them during the washing step (Sect. 3.5) to increase the concentration of cells. Also, the incubation can be extended to 1 h to increase the density.
9. The permeabilization of the cell membrane is extremely time sensitive. Allowing ethanol to be in contact with cells for more than 4 min leads to over-permeabilization, and cells will appear ghost-like in the phase contrast imaging. To be safe, we start the 4-min timer as soon as the first well is in contact with the ethanol. We start vacuuming the ethanol a minute before the 4-min timer is up, following the same order we applied the ethanol. Also, the surface should be completely dried. Otherwise, many cells appear floating or partially attached to the surface in the phase contrast images. The sample should be air-dried for 30–60 s before applying the next solution after the permeabilization.
10. It is well known that the fixation diminishes the intensity of the fluorescent proteins [35]. We have noticed that the formamide in the hybridization and washing steps has a similar effect. If the FROS signal does not look great after FISH, one can check the FROS signal after each step of the FISH procedure, by sealing the coverslip with a glass slide (i.e., jump to Step 12 in Sect. 3.9). We found that incubating the sample at 30°C, instead of 37°C, during prehybridization, hybridization, and washing helps to retain the FROS signal.
11. It is common to use a nail polisher to seal the coverslip [47]. We experienced that the fluorescence signal of Cy5 and Cy3B was much dimmer when the nail polisher was used for sealing [48]. The silicon-based dental sealant (Picodent) does not have this problem, and it is reversible. It can be cleanly removed using a razor blade and sealed again.
12. Representative images and smFISH analysis files are available for download from our GitHub site (https://github.com/sjkimlab/FISH2025).

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Conflict of Interest The authors have no conflicts of interest to declare that are relevant to the content of this chapter.

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