Video Article

Method for Labeling Transcripts in Individual *Escherichia coli* Cells for Single-molecule Fluorescence *In Situ* Hybridization Experiments

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Abstract

A method is described for labeling individual messenger RNA (mRNA) transcripts in fixed bacteria for use in single-molecule fluorescence *in situ* hybridization (smFISH) experiments in *E. coli*. smFISH allows the measurement of cell-to-cell variability in mRNA copy number of genes of interest, as well as the subcellular location of the transcripts. The main steps involved are fixation of the bacterial cell culture, permeabilization of cell membranes, and hybridization of the target transcripts with sets of commercially available short fluorescently-labeled oligonucleotide probes. smFISH can allow the imaging of the transcripts of multiple genes in the same cell, with limitations imposed by the spectral overlap between different fluorescent markers. Following completion of the protocol illustrated below, cells can be readily imaged using a microscope coupled with a camera suitable for low-intensity fluorescence. These images, together with cell contours obtained from segmentation of phase contrast frames, or from cell membrane staining, allow the calculation of the mRNA copy number distribution of a sample of cells using open-source or custom-written software. The labeling method described here can also be applied to image transcripts with stochastic optical reconstruction microscopy (STORM).

Video Link

The video component of this article can be found at https://www.jove.com/video/56600/

Introduction

Stochasticity is a fundamental and unavoidable aspect of gene expression and gives rise to cell-cell heterogeneity[1], both at the level of transcripts and proteins[2,3]. Quantifying the variability between cells under well-defined conditions offers a unique window into the basic processes that underlie gene expression and its regulation. One important source of cell-cell heterogeneity in bacteria takes place at the transcriptional level. Transcript numbers vary not only due to the stochasticity of transcription, but also to post-transcriptional processes such as regulation by small RNAs and RNAases[2]. One way of directly accessing this heterogeneity in a quantitative fashion is by fluorescently tagging individual transcripts of a given gene in smFISH. This methodology allows the detection and subcellular localization of particular RNA molecules in fixed, individual bacterial cells[4]. mRNAs are hybridized with a set of fluorescently-labeled ~20 base-long oligonucleotides that are designed to bind selectively to transcripts of interest[5,6]. Multiple labeling ensures detection above background fluorescence, and individual mRNA molecules appear as diffraction-limited spots under a fluorescence microscope[7] (**see Figure 1**). There are other approaches for labeling mRNA molecules, in which the complementary oligomer probes carry conjugated haptens (*e.g.*, biotin or digoxigenin) that are detected using secondary fluorescently-labeled reporter techniques[8].

There are other methods that provide quantitative information about transcripts, in addition to smFISH. Some, such as the Northern blot or quantitative PCR, probe the bulk and thus can measure neither the number of mRNA copies nor their position in individual cells. Therefore these methods are not suitable to quantify cell-to-cell variability. A recent image-based technique that allows for the quantification of both the copy number of RNAs within cells as well as their intracellular location, called multiplexed error-robust fluorescence *in situ* hybridization (MERFISH) has been developed. MERFISH is based on the assignment of a unique barcode consisting of a defined combination from a fixed number of fluorescently-labeled oligonucleotide probes. These barcodes are read out in sequential rounds of smFISH measurements, with photobleaching following each round of hybridization, thereby increasing throughput by two orders of magnitude[9,10]. This technique necessitates an automated fluid handling system and the proper design of the probe set.

The combination of multiple fluorescence labeling of individual transcripts, together with novel super-resolution techniques such as stochastic optical reconstruction microscopy (STORM)[11], enables a ten-fold increase in resolution in the subcellular localization of transcripts. In STORM, a suitable combination of fluorescent probes and imaging buffer allows for multiple cycles of fluorescence emission per probe molecule (blinking). STORM may also be used to image the *E. coli* transcriptome and observe genome-wide spatial organization of RNA, by labeling simultaneously all the transcripts of interest[12].

All the single-cell methods reviewed above are based on imaging transcripts in fixed cells. Hence, they do not provide any information regarding the kinetic properties of transcripts within cells. To follow transcripts in live cells[13], mRNAs can be labeled by the fusion of the gene of interest to an array of binding sites. These latter are then recognized by an RNA-binding protein, such as the bacteriophage MS2 coat protein, which is fused to a fluorescent protein such as the green fluorescent protein (GFP)[10,14,15].

Here we describe a method for labeling individual mRNAs with a set of fluorescently-labeled DNA probes, for use in smFISH experiments, in particular in *E. coli*. Furthermore, we show that the same labeling scheme may be used for STORM measurements with minor modifications.

Protocol

1. Probe Design

NOTE: This protocol uses commercially available oligonucleotide probes already tagged with fluorophores. The probes consist of a set of specific sequences complementary to a target mRNA, each probe being conjugated to a single fluorescent molecule. Alternatively, it is possible to attach fluorescent markers to probes, as described elsewhere[5,16].

1. Design smFISH probes using the Probe Designer[16] algorithm developed by Arjun Raj (van Oudenaarden Lab, Massachusetts Institute of Technology); sequences of smFISH probes used in this manuscript are listed in the table of materials.
   NOTE: The minimum number of probes in a set for a detectable signal is ~30. The precise number may vary according to the gene of interest, particularly if very short transcripts are measured.
2. Choose a dye that fits the optical setup (see table of materials).
   NOTE: The Cy3 dye or an optically equivalent dye with a low susceptibility to photo-bleaching is highly recommended. A candidate for dual labeling is Cy5 or an optically equivalent dye (see table of materials). Suitable dyes for STORM imaging of labeled mRNA are characterized by their capacity for multiple cycles of fluorescence emission. Some smFISH dyes can be used for STORM imaging (see table of materials). To image two (or more) transcripts corresponding to different genes in the same cell, one should choose oligonucleotide probes conjugated to dyes whose spectra have little or no overlap.

2. Reagent Preparation

NOTE: It is important to keep the samples RNAse-free by using RNase-free consumables such as filtered pipette tips and tubes, and to keep any working environment clean. In order to avoid degradation of the target transcripts, all reagents used following cell fixation must be nuclease-free (see table of materials) or diethylpyrocarbonate (DEPC)-treated and sterilized.

1. **Buffers for smFISH**
   1. Prepare RNase-free 1x PBS (phosphate buffered saline, solution with a phosphate buffer concentration of 0.01 M and a sodium chloride concentration of 0.154 M, (pH 7.4)). Dilute RNase-free 10x PBS (see table of materials) in nuclease-free water (see table of materials) at a 1:10 v/v ratio.
      1. Alternatively prepare DEPC-treated 1x PBS.
   2. Prepare RNase-free 2x SSC (saline-sodium citrate buffer, 0.3 M NaCl in 0.03 M sodium citrate, (pH 7.0)). Dilute RNase-free 20x SSC (see table of materials) in nuclease-free water at a 1:10 v/v ratio.
      1. Alternatively, prepare DEPC-treated 2x SSC.
2. **Oligonucleotide probe stock solution.**
   1. Re-dissolve the dried oligonucleotide probe blend in 200 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) to create a probe stock of 25 µM. Mix well by pipetting up and down, and then vortex and centrifuge briefly.
   2. To avoid freeze and thaw of the stock solution tube, make several aliquots of the stock solution, corresponding to the amount expected to be used in a single experimental run. Store the stock solution at -20 °C or below and protect from light.
      NOTE: A probe set of 5 nmol can provide up to 250 hybridization reactions.
3. **Hybridization buffer (following Skinner *et al.*[7]):**
   1. Add 5 mL nuclease-free water to a 50 mL polypropylene conical-bottom tube. Add 1 g of dextran sulfate to the water and dissolve it by vigorous vortexing. Mix the contents in an orbital shaker until bubbles disappear (~30 min).
   2. Add to the solution 3,530 µL of deionized formamide, 10 mg of *E. coli* tRNA, 1mL of 20x SSC, 100 µL of 200 mM vanadyl-ribonucleoside complex and 40 µL of 50 mg/mL BSA. Bring the final volume to 10 mL by addition of DEPC-treated water or nuclease-free water, and vortex the solution until it is homogenous.
      NOTE: To avoid freeze and thaw of the solution, make several aliquots of the stock solution, corresponding to the amount expected to be used in a single experimental run. Store the stock solution at -20 °C. Be sure to let the formamide warm to room temperature before opening the bottle. To reduce the background, it is suggested to calibrate the optimal formamide concentration (10 - 40% v/v ratio).
      Caution: WARNING! Formamide is highly toxic and a known teratogen. Avoid contact with the eyes or skin, inhalation or ingestion. Handle it under a fume hood while wearing a lab coat and protective gloves.
   3. Sterilize the solution by passing it through a 0.22-µm filter. Keep in aliquots and store at -20 °C up to one year.
4. Prepare Fixation buffer (4.6% formaldehyde in 1x PBS) by adding 37% formaldehyde (v/v) to 1x PBS (see 2.1.1) at a 1:8 v/v ratio and vortex.
   CAUTION: WARNING! Formaldehyde is highly toxic and a known carcinogen. Handle it under a fume hood while wearing a lab coat and protective gloves.
5. Prepare wash buffer for smFISH (20% formamide in 2x SSC) by adding deionized formamide to 2x SSC (see 2.1.2) to a 1:4 v/v ratio and vortex.

6. Prepare imaging buffer for smFISH by adding 5 mM cysteamine and oxygen scavengers (7 µM glucose oxidase, 56 nM catalase, and 10% glucose (w/v)) to wash buffer (20% formamide in 2x SSC)
7. **Buffers for STORM**
    1. Prepare Buffer A by adding 50 mM Tris-HCl and 10 mM NaCl to nuclease-free water.
    NOTE: Store at RT for years.
    2. Prepare Buffer B by adding 50 mM Tris-HCl, 10 mM NaCl, and 10% w/v glucose to nuclease-free water.
    NOTE: Store at 4 °C for up to a year.
    3. Prepare MEA buffer by dissolving 77 mg cysteamine in 1 mL of buffer A (makes a 1 M solution).
    NOTE: It can be stored at 4 °C for 1 month.
    4. Prepare Gloxy buffer by adding 8,440 AU (arbitrary units) glucose oxidase and 70,200 AU catalase to 1 mL buffer A.
    NOTE: Makes 50x stock solution, it can be stored at 4 °C for 1 month.
    5. Prepare imaging buffer for STORM by adding 50 mM of MEA buffer and 1x Gloxy buffer to buffer B.
    NOTE: The imaging buffer for STORM is composed of 5 mM cysteamine, oxygen scavengers (7 µM glucose oxidase and 56 nM catalase) in 50 mM Tris with 10 mM NaCl, and 10% glucose at pH 8.0. Prepare just before imaging, it can be stored and used at RT for approximately 2 h.

3. Sample Fixation

1. Grow bacterial cells (*e.g.*, *E. coli* MG1655) in growth media of choice (*e.g.*, LB medium) overnight inan orbital shaker at 260 rpm and 37 °C.
NOTE: To determine the background due to non-specific binding of the probes, measurements in a strain in which the gene of interest has been deleted should be conducted, following the same procedures (see Δ*galk* in **Figure 1** and Δ*sodB* in **Figure 2**).
2. Dilute the overnight culture 1:100 in fresh medium and measure its optical density (OD_{600}) in a spectrophotometer every ~1 h, up to a value of 0.2 - 0.4; a 4 mL culture should be sufficient.
NOTE: If the chosen microscope does not have phase contrast or differential interference contrast capabilities, *E. coli* outer membranes can be labeled with 2 µM lipophilic fluorescent marker (see table of materials), which should be added to the bacterial suspension 20 min prior to fixation[17]. Fixation and further treatment are accomplished as described below. Care must be taken to choose a fluorescent marker having a different emission spectrum, to minimize the spectral overlap with the labeled-probe set.
3. Pellet cells by centrifugation for 5 min at 4,500 g at 4 °C. Remove the supernatant from all tubes.
4. Add 1 mL of 1x PBS to each tube and resuspend the pellet to minimize cell-to-cell aggregation. Then add 4 mL of fixation buffer and vortex gently.
5. Incubate samples in an orbital shaker at 260 rpm for 30 min at 24 °C.
6. Pellet cells by centrifugation for 10 min, 1,000 g, 4 °C. Remove supernatant. Add 1 mL 1x PBS.
7. Transfer suspensions to 1.8 µL conical-bottom micro-centrifuge tubes (see table of materials) and repeat step 3.6 twice.

4. Sample Permeabilization

1. Remove supernatant very carefully. Use small volume pipettes, if necessary, to remove all the liquid outside the pellet.
2. Resuspend in 300 µL DEPC-treated water or nuclease-free water. Add 350 µL high grade absolute ethanol (99%) and mix gently by inverting the tube. Add another 350 µL of ethanol and mix again, to reach a final ethanol concentration of 70% (v/v ratio).
CAUTION: WARNING! Ethanol is flammable.
3. Rotate the sample with a tube rotator at 20 rpm for 1 h at room temperature (RT), or at 4 °C overnight. Samples may be stored at 4 °C up to one week.

5. Hybridization

1. Pellet cells by centrifugation for 7 min, 750 x g, 4 °C. Remove supernatant.
2. Add 1 mL 20% wash buffer to the samples and leave standing for several minutes, or pipette to dissolve the pellet completely.
3. Pellet cells by centrifugation for 7 min, at 750 x g, 4 °C.
4. Remove the supernatant very gently by pipetting. Use small-volume pipettes if necessary to remove all the liquid outside the pellet.
5. Add the oligonucleotide probe set stock solution to the hybridization buffer aliquot so that the final oligonucleotide concentration is 250 nM; vortex vigorously.
NOTE: One can label two or more different target mRNAs at once in the same cell. Add probe sets of both targets to the hybridization buffer. Care must be taken to choose dyes having different emission spectra to minimize the spectral overlap (*e.g.*, Cy3 and Cy5). Make sure that samples intended for STORM imaging are hybridized with an oligonucleotide probe set conjugated with a suitable dye for STORM (*e.g.*, in table of materials).
6. Suspend samples (see step 5.4) in 50 µL hybridization buffer that contains the oligonucleotide probes, while avoiding the generation of bubbles (the solution is quite viscous).
7. Leave samples overnight on a hot block at 30 °C in dark.
NOTE: Following this, samples may be stored for up to 6 months at 4 °C.

6. Washing

1. Use one new microcentrifuge tube for each sample to image. Add 1 mL wash buffer.
2. Add 10-15 µL from the hybridized samples to the new tube that contains the wash buffer and mix/vortex.
3. Pellet cells by centrifugation for 7 min, 750 x g at 4 °C. Remove supernatant.
NOTE: To reduce background, incubate for 1 h at 30 °C.

4. Wash twice more (resuspend in 1 mL wash buffer, centrifuge and remove pellet).
5. Resuspend in 25 µL wash buffer.
   NOTE: In order to reduce photo-bleaching one can add to the wash buffer an oxygen scavenging system (2.6) to improve dye stability in single-molecule fluorescence experiments[6].

7. Preparation of Samples for Imaging

NOTE: Cells need to be immobilized in order to allow proper imaging under a fluorescence and phase-contrast microscope. The following methods can be used to prepare samples in which different fields of view can be imaged at different focal planes.

1. **Agarose gel preparation**
   1. Add 150 mg agarose to 10 mL 2x SSC buffer. Do not add 2x SSC to agarose, or else it will not dissolve properly.
   2. Microwave for 10-15 s each time until large bubbles begin to form. Set aside to cool for a few minutes.
   3. Place a separating silicone frame on the slide so that the slide's center remains exposed.
   4. Place a 10 mm wide (1-2 mm thick) rigid ring in the center of the slide and deposit a small amount of gel in it, for sealing. Wait a few minutes for it to harden.
   5. Add more gel until a high dome shape is formed. Wait 10 min for it to harden, and remove the ring (using fine tweezers or any other method).
   6. Deposit ~10 µL of washed bacterial cells (see 6.5) on the gel and seal with a #0 coverslip slide.

2. **Chamber sample preparation for smFISH**
   1. Alternatively (to step 7.1) prepare samples for imaging by immobilizing the washed bacterial cells (see 6.5) on poly-D-lysine-coated glass bottom disposable plastic petri dishes.
   2. Incubate the samples in the dark, at room temperature, overnight before visualization to allow adhesion of the cells to the surface. Before visualization wash once with wash buffer (.5); keep the sample wet with wash buffer(2.5) or imaging buffer for smFISH (2.6).

3. **Chamber Sample Preparation for STORM**
   1. Prepare samples for STORM for imaging on poly-D-lysine-coated glass bottom disposable plastic petri dishes.
   2. Incubate the samples in the dark, at RT, overnight. Before visualization wash once with wash buffer for smFISH (2.5); and add imaging buffer for STORM (2.7.5).

8. Transcript Visualization

1. Image cells with a wide-field fluorescence microscope equipped with a motorized stage (see table of materials).
   NOTE: For STORM modification, use cells labeled with a dye capable of multiple cycles of fluorescence emission. Image the sample using 3D super-resolution imaging system (*e.g.*, see table of materials).
2. Use (recommended) a 60-100x N.A >1.3 oil immersion phase contrast objective lens with a strong light source, such as a mercury or metal-halide lamp; LED-based light sources are also recommended.
3. Use a standard cooled CCD camera, ideally optimized for low-light level imaging rather than speed (we use a camera with 16 µm pixel size) (see table of materials).
   NOTE: The use of a cooled EMCCD (electron multiplying charge coupled device) camera CCD camera is necessary to reduce thermal noise that prevents detection, particularly of single molecules.
4. Use filter sets appropriate for the fluorophores chosen.
5. To determine properly cell boundaries, acquire images of different fields of view in each sample either with phase contrast optics, or by labeling fluorescently the outer cell membrane. Acquire images at different focal planes (z-stack) for all channels (usually 13 slices separated by 250 nm are taken).
   NOTE: The motorized stage of the microscope and the filter sets should be controlled by commercial (see table of materials) or in-house software that can capture a stack of images in sequence.

9. Data Analysis

1. Convert the image stacks to a suitable format for data analysis.
2. Estimate the copy number of the target mRNA from the total intensity of fluorescent foci in the cell, rather than from counting discrete 'spots' as in other currently available protocols.
3. Carry out image analysis with open-source software[18] or with a custom-built program. For details of data imaging and analysis see Skinner *et al.*[7]

Representative Results

We carried out smFISH measurements of galK and sodB transcripts in *E. coli* cells. The transcripts were hybridized with a set of specific sequences complementary to the target sequence, each probe being conjugated to a single fluorescent molecule (see table of materials). Fluorescence and phase contrast images of MG1655 wild-type *E. coli* strain (WT) or a JW0740 (Keio collection)[19] galK-deleted strain (Δ*galK*) were exposed to 2 mM D-fucose are shown in **Figure 1A** and **1B**. Cells were fixed at least 3 h after exposure to D-fucose. The panels in **Figure 1A** (top) show one frame out of 13, corresponding to the height at which cell contours are in focus, representing the level of *galK* induction in response to variable extracellular D-fucose concentrations. Spots within cells in the fluorescence images correspond to either single or few galk transcripts, and the determination of the transcript number in each cell is carried out by quantifying localized fluorescence. Numerous spots are seen in most cells, when 2 mM D-fucose is added to the bacterial culture, whereas a few spots are seen in the absence of extracellular D-fucose. In the Δ*galK* strain no spots are observed over the background as anticipated. Surface labeling with lipophilic fluorescent marker is shown in **Figure 1B** (bottom).

In addition, we imaged the *sodB* transcripts in *E. coli* bacterial culture grown in LB medium in the logarithmic growth phase. Fluorescence and phase contrast images of smFISH in WT or a JW1648 (Keio collection)[19] sodB-deleted strain (Δ*sodB*) are shown in **Figure 2A**. The panels in **Figure 2A** (top) show one frame out of 13, corresponding to the height at which cell contours are in focus, representing the level of *sodB*. In the Δ*sodB* strain, no spots are observed over the background, as anticipated.

The same cultures were imaged by STORM (top) and the perimeters of the cells were determined with surface labeling using a lipophilic fluorescent marker to illustrate the localization of *sodB* within the cells. Staining the membrane allows accurate positioning of transcripts in the cell (**Figure 2B**). The Δ*sodB* strain was taken as the background signal and to determine the minimal cluster size (see **Figure 2B**). The mean transcript number of smFISH[2] and STORM is comparable.

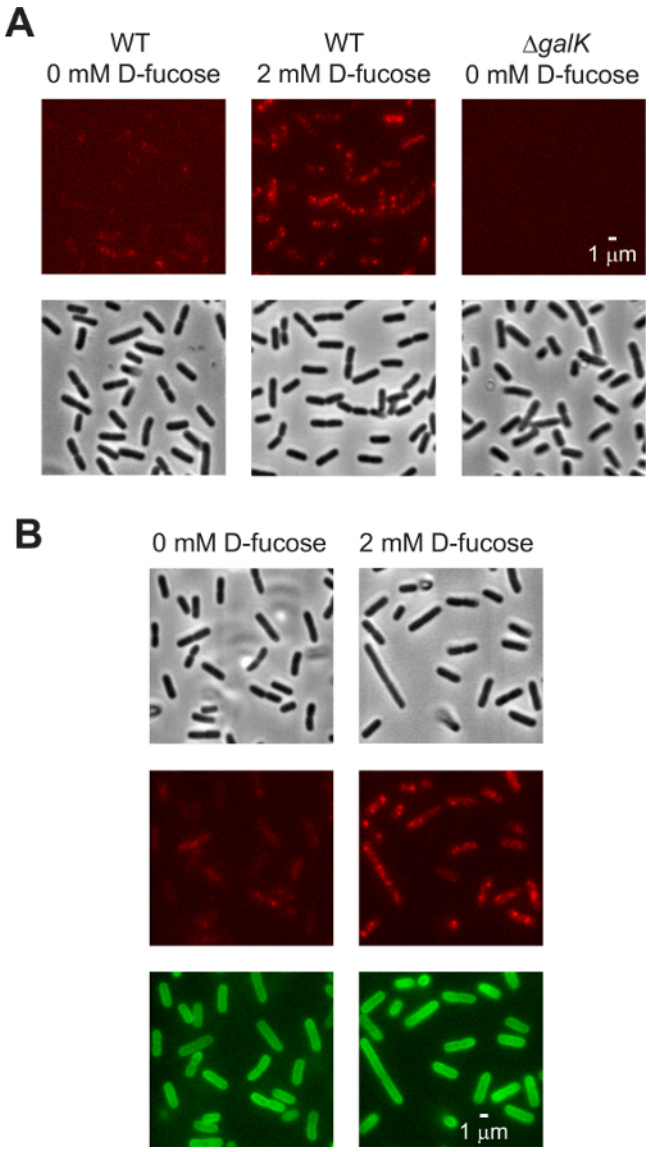

Figure 1: Effects of D-fucose on galK transcripts visualized using smFISH. (A) Top: smFISH images of fluorescence of galK mRNA in individual *E. coli* cells. An MG1655 wild-type strain (WT) or a JW0740 (Keio collection) *galK*-deleted strain (Δ*galK*)[19] were grown with or without 2 mM D-fucose. The galK transcripts were hybridized to complementary probes conjugated with a dye optically equivalent to Cy3 (see table of materials). Bottom: same fields of view in phase-contrast. **(B)** WT cells were grown with or without 2 mM D-fucose as above and labeled with 2 μM of a lipophilic membrane dye (see table of materials) for 20 min prior to fixation. Top: fluorescence images of galK mRNA using smFISH. Images were taken with a microscope controlled by a commercial software using a 100× N.A 1.45 oil immersion phase contrast objective lens (see table of materials) and an EMCCD camera (see table of materials). The filters used were as described in the table of materials. A phase contrast image was acquired followed by a z-stack of 13 slices and 250 nm spacing of fluorescent images with 2 s integration time for each slice. Middle: same fields of view with phase-contrast imaging. Bottom: cells labeled with lipophilic fluorescent markers, with appropriate filters described in the Materials Table. Please click here to view a larger version of this figure.

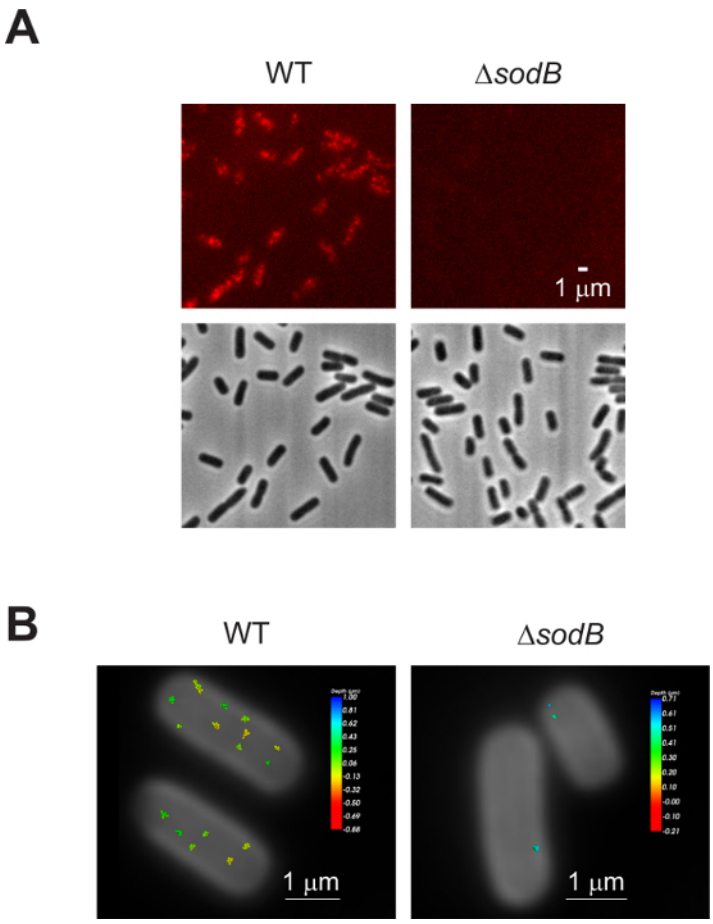

Figure 2: Imaging of sodB transcripts labeled with the same probe set in smFISH and STORM. (A) Top: smFISH images of fluorescence of sodB mRNA in individual *E. coli* cells. An MG1655 wild-type strain (WT) or a JW1648 (Keio collection) *sodB*-deleted strain (Δ*sodB*)[19] were grown in LB medium. The *sodB* transcripts were hybridized to complementary probes conjugated with a dye optically equivalent to Cy5 (see table of materials). Bottom: same fields of view in phase-contrast. **(B)** Overlay of fluorescent molecules (individual colored dots) localized by STORM (670 nm emission) and a snapshot of cell membranes in the same field of view. The cells were labeled with a lipophilic dye, excited with an argon laser at 488 nm at 1% of maximal power (0.05 kW/cm^2) with an emission peak at 510 nm and imaged with the appropriate filter (see table of materials). WT and *sodB* cells were grown as described above and labeled with a 2 μM lipophilic membrane dye (see table of materials) for 20 min prior to fixation. Super-resolution images were recorded with a commercial microscope (see table of materials). Transcripts labelled with a dye optically equivalent to Cy5 were localized using an excitation laser at 647 nm in an imaging buffer for STORM. Images were recorded using a 60x, NA 1.2 water immersion objective (see table of materials) and an EMCCD camera (see table of materials) with gain set at 50, frame rate at 50 Hz, and maximal power of 647 and 405 nm lasers set at 5 and 0.05 kW/cm^2, respectively. The total number of frames acquired was 8,000. Data was analysed using commercial software (see Materials Table). Please click here to view a larger version of this figure.

Discussion

We have measured in our laboratory the transcript number of different genes in *E. coli* cells using the smFISH method[2]. In brief, this procedure consists of the following steps: cell fixation, permeabilization of membranes to allow for probe penetration, probe hybridization, and sample imaging using a standard fluorescence microscope. This procedure is based on previously published ones with some modifications[6,7,16]. It has been previously reported that smFISH requires that the number of oligonucleotide probes lie in the range 48-72, in order to achieve a signal lying well above the background[7]. We have shown that this number may be actually smaller[20], depending on the gene sequence of interest, the optical setup, and the specific experimental conditions.

Each probe should be 17-22 nucleotides long, with an inter-probe separation of at least two nucleotides and a GC content of ~45%, in order to reduce the effects of non-specific, off-target binding. Some probes may fail to bind a target, and the overall efficiency of binding can be optimized by modifications of experimental procedures such as the conditions of fixation and hybridization[21]. An important factor that must be taken into account is the choice of fluorophores. It is highly recommended to label the probes with a dye with low susceptibility to photo-bleaching. Photo-bleaching minimization can be achieved by optimization of exposure times, illumination sources, and integration times of image acquisition, and by the addition of an oxygen scavenging system.

The extent of non-specific binding events should be assessed by carrying out smFISH experiments with cells from strains in which target genes are deleted, for example from the Keio collection[19]. If the background signal is high, the number and duration of washing steps should be increased, or cells should be incubated in wash buffer at 30 °C for 1 h and then washed twice. Furthermore, to reduce the background, it is suggested to optimize the formamide concentration (10-40% v/v ratio) in the hybridization and the wash buffers. Increasing the formamide

concentration reduces nonspecific binding, but may also reduce the binding of probes to the target mRNA. During the hybridization step, it is important to remove the supernatant entirely; a dilute hybridization buffer may lead to decreased labeling efficiency. In addition, target RNAs must be sufficiently long in order to obtain a well-localized spot above background. Recent improvements in signal to-noise ratios and binding specificity through backbone modification of the probes now make it possible to detect shorter RNA fragments, such as eukaryotic microRNAs or bacterial small non-coding RNAs[2,10]. We recommend that the mean copy number obtained by smFISH should be validated with quantitative PCR[7,16].

We have shown in this manuscript that this method can be modified with minor changes to study the localization of transcripts in *E. coli* with higher optical resolution using stochastic optical reconstruction microscopy (STORM)[11].

In summary, smFISH is a versatile method for RNA illumination that allows the direct measurement of the cell-cell variability in transcript number and the localization of target transcripts in the cell, in both eukaryotes and prokaryotes. It provides quantitative information about basic processes of gene expression and can be easily implemented for STORM imaging.

Disclosures

The authors have nothing to disclose.

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