Measuring mRNA copy number in individual *Escherichia coli* cells using single-molecule fluorescent *in situ* hybridization

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We present a protocol for measuring the absolute number of mRNA molecules from a gene of interest in individual, chemically fixed *Escherichia coli* cells. A set of fluorescently labeled oligonucleotide probes is hybridized to the target mRNA, such that each mRNA molecule is decorated by a known number of fluorescent dyes. Cells are then imaged using fluorescence microscopy. The copy number of the target mRNA is estimated from the total intensity of fluorescent foci in the cell, rather than from counting discrete 'spots' as in other currently available protocols. Image analysis is performed using an automated algorithm. The measured mRNA copy number distribution obtained from many individual cells can be used to extract the parameters of stochastic gene activity, namely the frequency and size of transcription bursts from the gene of interest. The experimental procedure takes 2 d, with another 2–3 d typically required for image and data analysis.

INTRODUCTION

Development of the protocol

Fluorescent *in situ* hybridization (FISH) has been used to detect individual mRNA molecules of a gene of interest and measure their copy number in individual eukaryotic cells[1–3]. This procedure is referred to as single-molecule FISH (smFISH). Different variants of the method exist[4–6]. In one particularly popular protocol, introduced by Raj *et al.*[4], ~20-base-long nucleotides are used as probes. Each probe is labeled with a single fluorescent dye molecule, and a set of ~50 different probes are hybridized to the mRNA of interest. As a result, a single mRNA molecule produces enough signal to be easily detectable as a diffraction-limited spot under the fluorescence microscope. Counting these spots provides an estimate of mRNA copy number in the cell[3,4,6,7].

We recently adapted smFISH for measuring the number of mRNA copies from a gene of interest in individual *E. coli* cells[8,9]. Examining the copy-number statistics in a population of cells then allows us to extract the parameters of stochastic gene activity, namely how often transcription bursts occur (burst frequency) and how many mRNA molecules are produced within each burst (burst size)[8–10]. This procedure can be repeated for different endogenous genes, under different growth conditions and expression levels[9]. Our protocol is derived from the one by Raj *et al.*[4] in terms of probe design and biochemical procedures used. However, we diverge from other smFISH protocols[4,6] in two important aspects.

The first difference is that the estimation of mRNA number in the cell is not achieved by counting discrete spots, but instead relies on quantifying localized fluorescence. Owing to the optical properties of a standard fluorescence microscope, a single mRNA molecule creates an image of size ~250 nm in the horizontal plane[11,12]. Thus, two molecules that are closer than that distance will overlap each other and will not be detectable as separate spots. This distance is equivalent to a concentration of ~10 nM or ~10 molecules in one *E. coli* cell. For comparison, the induced lactose promoter produces ~50 mRNA molecules per cell[9]. Thus, counting spots will not allow us to reliably measure mRNA levels for a highly expressed gene in *E. coli*, because many of the apparent spots will consist of more than one mRNA. Our solution is instead to measure the number of bound probes on the basis of the total fluorescence intensity (photon flux) of the spots, without requiring that individual mRNAs appear as separate spots. By performing a calibration step, the total intensity of spots in the cell can then be converted to the number of target mRNAs. This procedure is inspired by the method we previously developed for counting mRNAs in live cells using the MS2-GFP labeling scheme[10,13]. It involves the development of automated image and data analysis algorithms, as described below.

A second difference from most previous protocols[5,6,14] is that all biochemical steps (fixation, permeabilization, washes and hybridization) are performed in test tubes rather than on microscope slides. We reasoned that quantitative biochemical measurements require perfect mixing and uniformity of conditions. In contrast, cells attached to a slide are subject to nonuniform conditions, sometimes resulting in spatially inhomogeneous labeling[15]. Uniformity is especially crucial when one is aiming to accurately quantify cell-to-cell variability, as one must avoid increasing any experimental heterogeneity. We therefore developed the tube-based protocol presented here.

Applications of the method

The protocol allows measuring the absolute number of endogenous mRNA molecules from a gene of interest in individual *E. coli* cells. The dynamic range of the measurement is from <1 to ~100 molecules per cell[9] (**Fig. 1**). The estimated precision of the measurement is <1 (i.e., single-molecule resolution) at low mRNA levels (**Fig. 1d**). Under the assumption that the labeling

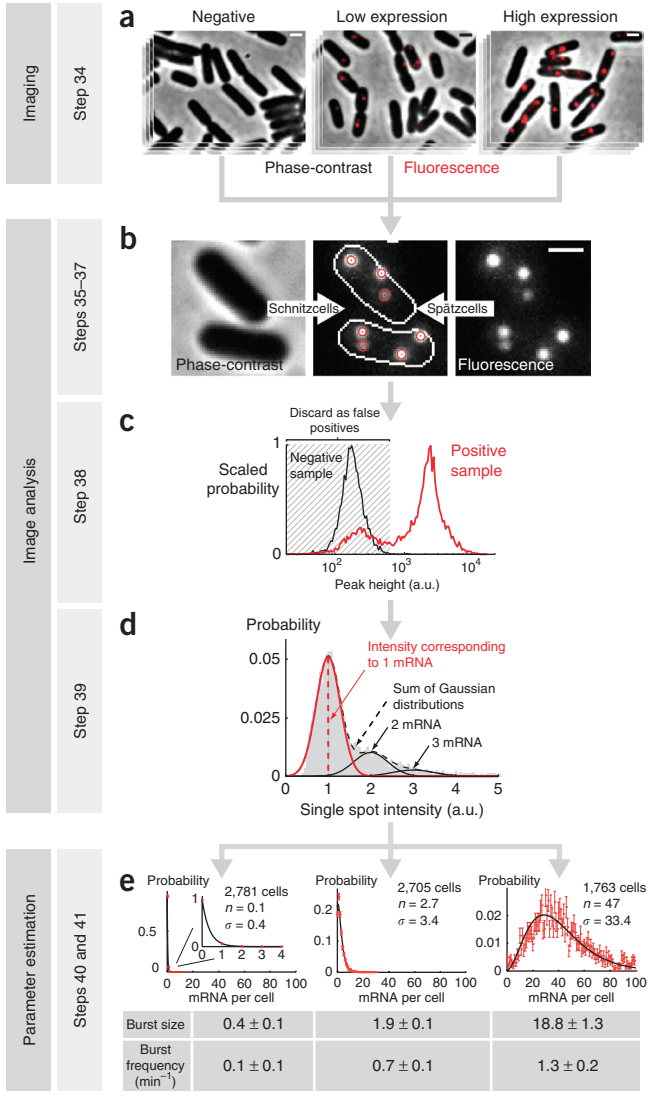

Figure 1 | Experimental procedure. (**a**) Image acquisition. Phase-contrast and fluorescence images are acquired for each sample. Multiple focal planes (*z* positions) are imaged to allow precise fluorescence detection throughout the depth of the cell. (**b**) Cell and spot segmentation. The positions of individual cells and fluorescent foci (spots) are identified using custom MATLAB codes. Cell recognition uses as input the phase contrast images, and is performed using the Schnitzcells program (see Equipment). Fluorescent spots are identified from the stacks of fluorescence images, using the Spätzcells program developed in our laboratory. (**c**) Discarding false positives. False-positive spots, which are the result of probe binding to nontarget RNA, are discarded after examination of the histogram of peak height (spot intensity maximum) in a negative sample. (**d**) Identifying fluorescence intensity of one mRNA. The spot intensity corresponding to a single mRNA molecule is identified by examining the histogram of single spot intensities in a low-expression sample, where individual mRNAs are spatially separable. (**e**) Converting fluorescence intensity into mRNA numbers and extracting kinetic parameters. The one-mRNA intensity value is used to convert the total spot intensity in any cell to the number of target mRNA molecules. By measuring mRNA numbers in >1,000 cells per sample, the population mean and variance are estimated. The copy-number histogram is fitted to a simple model of transcription kinetics. The parameters of the fit are used to calculate the frequency and size of transcription bursts. Scale bars, 1 µm. a.u., arbitrary units.

and detection of individual mRNA molecules are statistically independent[3], this translates to an error of a few percent at the higher end of the measurement range. Although other factors may increase the measurement error, the low error estimation is supported by the good agreement that smFISH data show with quantitative PCR (qPCR)[8,9], as well as with theoretical predictions[8,9]. In a typical experiment, mRNA numbers from >1,000 individual cells are measured for a given sample, and the population mean $\langle n \rangle$ and variance σ^2 are estimated[8,9]. The copy-number histogram is fitted to a simple model of transcription kinetics[2,16,17]. The parameters of the fit are used to calculate the frequency and size of transcription bursts[8–10]. We have previously used the above procedure to characterize the activity of 20 promoters under different growth conditions[8,9].

A number of variations and extensions to the application above are possible. The mRNA numbers for multiple genes can be measured simultaneously by using probe sets labeled with fluorophores of distinct spectra. We have used multicolor smFISH to examine the coexpression of two genes in *E. coli*[8]. The detection of three mRNA species simultaneously has been reported in eukaryotic systems[4]. This should also be achievable in bacteria by following the same considerations regarding probe design and optical setup[4,6,12]. Achieving a higher degree of multigene combination would require more advanced spatial or spectral multiplexing methods[12,18].

In addition to counting mRNA, smFISH has been used in eukaryotes to examine the spatial organization of RNA molecules in the cell[6,19]. A similar investigation can be performed in bacteria. For example, the site of active transcription from the gene can be distinguished from the individual, freely diffusing mRNAs on the basis of the morphological properties of the corresponding fluorescent foci (not shown). Characterizing the positions of these two mRNA species can shed light on the spatiotemporal life history of mRNA, a subject of ongoing debate[20–24].

Finally, we have successfully applied the image analysis component of this protocol—namely spot recognition and calibration of fluorescence to mRNA numbers—when performing smFISH in higher organisms using the protocol of Raj *et al.*[4]. The same image analysis algorithms can also be used, *mutatis mutandis*, for estimating mRNA numbers from live-cell images in which mRNA is labeled using the MS2 coat protein fused to a fluorescent protein[10,13,25].

Comparison with other methods

In terms of quantifying relative expression levels (i.e., comparing two samples), smFISH-based measurements show good agreement with the results of qPCR over approximately three orders of mRNA levels[8,9]. The comparison between the two methods can be extended to absolute numbers by calibrating the qPCR data, using either external (*in vitro*–transcribed RNA) or internal (e.g., ribosomal RNA) controls[10,26]. However, we have not performed such a comparison. In higher organisms, smFISH data have also been compared with results obtained by RNA-sequencing (RNA-seq)[27], but again we are not aware of a similar comparison in bacteria.

In terms of single-cell measurements of mRNA numbers, two other smFISH protocols (both performed on microscope slides rather than in test tubes) have been reported in bacteria. Maamar *et al.*[14] used smFISH to quantify mRNA levels in individual *Bacillus subtilis* cells. Taniguchi *et al.*[5] labeled mRNA in *E. coli* using a single

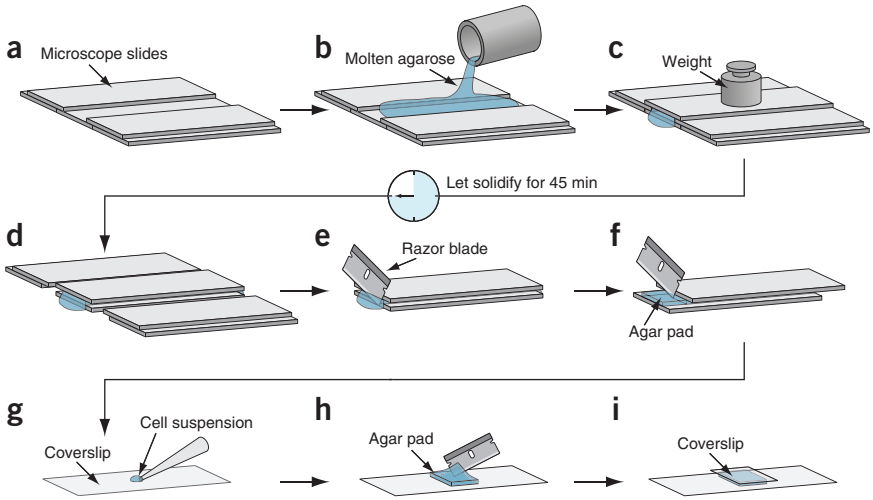

Figure 2 | Preparation and use of agarose pads. (**a**) Stack five microscope slides on a leveled surface. (**b**) Pour the molten agarose solution onto the slides. (**c**) Cover the agarose with the remaining slide, placing a weight on top. Let the agarose solidify for 45 min at room temperature. (**d**) Remove the four slides from the sides of the agarose pad, leaving the top and bottom slides for easy storage and handling. (**e**) Remove the excess agarose from the slides with a razor blade. (**f**) For use in imaging, carefully move the slides, exposing the agarose, and excise a 1 × 1 cm agar pad with a razor blade. (**g**) Pipette 2 µl of the cell suspension onto the center of a 24 × 50 mm coverslip. (**h**) Lay the agarose pad slowly on top of the cell suspension droplet with the razor blade. (**i**) Cover the pad with a 22 × 22 mm coverslip.

probe carrying a single fluorophore per mRNA target. In both these protocols, mRNA numbers were estimated by spot counting. This approach probably limits the measurement accuracy at high mRNA numbers. Consistent with this hypothesis, no mRNA levels higher than about ten molecules per cell were reported in these studies. This is in contrast to our work, in which such higher values were repeatedly measured[9].

Finally, mRNA can also be labeled in individual live cells using a fusion of fluorescent protein to an RNA-binding protein (such as the MS2 coat protein), and fusing an array of the corresponding binding sites to the gene of interest[20,28,29]. We previously used this approach to follow the kinetics of mRNA production in *E. coli*[10]. This labeling scheme is highly perturbative to gene function[10]. Thus, a direct comparison of expression level with genetically unperturbed cells is not possible. Nevertheless, the typical size of transcription bursts detected using live cell imaging is similar to that found using smFISH[8–10].

Experimental design

In terms of probe design and biochemical procedures, our protocol is directly based on that of Raj *et al.*[4]. A set of ~50 probes, each ~20 bases long, are designed against the transcript of interest (see Reagent Setup). The probes can be purchased prelabeled, with a single fluorescent dye molecule on the 3′ end of each oligonucleotide. Alternatively, amine-modified oligos can be purchased and then fluorescently labeled in the lab and purified by ethanol precipitation (PROCEDURE Steps 1–11). We have successfully used as few as 48 probes and as many as 72 probes per gene.

Each experiment is performed using the strain of interest, a low-expression control sample and a negative control sample (i.e., a strain lacking the target mRNA). The cells to be studied are grown overnight (Step 12). The next day, cells are grown to mid-log phase (Steps 13 and 14), fixed (Step 16) and permeabilized (Step 17). The cells are then mixed with the labeled probes and hybridized overnight (Steps 19–23). The next morning, the cells are washed to remove nonhybridized probes (Steps 24–30), and they are finally resuspended in imaging buffer (Step 31). These steps are all performed in tubes to guarantee that all cells experience a uniform environment and to promote perfect mixing.

To acquire data, cells are placed between a coverslip and a thin agar slab (**Fig. 2**, Step 32) and imaged using both phase contrast and epifluorescence microscopy (**Fig. 1a**, Steps 33 and 34). Images are acquired using a high-quantum-yield, cooled charge-coupled device (CCD) camera. Multiple positions on the coverslip, providing data for >1,000 cells from each biological sample, are taken. Imaging is performed at multiple focal planes (*z* positions) to allow high-resolution coverage of the cell depth (~1 µm).

Next, images are analyzed using custom MATLAB codes in order to obtain the positions of individual cells and fluorescent foci (**Fig. 1b**, Steps 35–37). Cell recognition uses as input the stacks of phase contrast images, and is performed using the Schnitzcells program[30]. Fluorescent foci (spots) are identified from the stacks of fluorescence images, using the Spätzcells program, developed in our lab for that purpose.

The conversion of fluorescent foci intensity to mRNA numbers is achieved in a few steps (**Fig. 1c–e** and Steps 38–40). First, false-positive spots, which are the result of probe binding to nontarget RNA, are discarded after examination of spot statistics in a negative control sample. Next, the spot intensity corresponding to a single mRNA molecule is identified by examining the histogram of spot intensities in a low-expression control sample, where individual mRNAs are spatially separable. Finally, this single-mRNA intensity value is used, in a sample of an unknown expression level, to convert the total spot intensity in each cell to the estimated number of target mRNAs.

By measuring mRNA numbers in >1,000 cells, the population mean and variance are estimated. The copy-number histogram is fitted to a simple theoretical model for stochastic gene activity (**Fig. 1e**, Step 41). The fitting parameters are used to estimate the rate and size of transcription bursts from the gene. mRNA half-life, which is required for this calculation, can be measured using standard methods[8,9].

Limitations

As described above, the purpose of the protocol is to obtain a precise estimate of the number of mRNA molecules from a gene of interest in individual cells, and to use copy-number statistics from a population of cells to extract the underlying parameters of

stochastic gene activity. Achieving this goal can be hindered by a number of factors. First, the calibration of fluorescence intensity to the number of mRNA molecules requires the use of a completely negative (i.e., no mRNA of interest present) control sample in order to discard false-positive spots, as well as a low-expression control sample, in which most mRNAs are discernible as individual spots. There may be cases in which either of these controls is not available, for example, if the gene of interest is essential and cannot be deleted. In those cases, calibration is harder to perform and may result in lower accuracy.

We also note that the accuracy of the measurement is estimated mainly using internal controls, namely by assessing the error in identifying the single mRNA peak in the spot intensity histogram (**Fig. 1d**). Additional external controls are potentially very useful. In particular, it can be helpful to compare spot intensity with the fluorescence of individual probes in order to estimate the probe hybridization efficiency[6] or to compare smFISH-based mRNA levels with the results of qPCR[8,9]. However, in our hands, these added measurements are more technically challenging than the smFISH measurements themselves and are harder to render quantitative, and thus they are limited as standards against which to compare the smFISH data.

The estimation of gene activity parameters—frequency and size of transcription bursts—is performed using the mRNA copy-number histogram in a population of cells. The validity of this procedure depends on an assumption of steady state, i.e., that mRNA production and degradation in the cells are balanced[2,8,9,16,17]. This assumption is most easily fulfilled during exponential cell growth. In some cases, however, gene activity *outside* steady state is of interest, such as in the case of the transient response to an external stimulus such as a drug[31,32]. In that case, mRNA numbers can be measured in samples taken at different time points, with a temporal resolution of ~1–2 min (limited by sample handling times). The analysis of cell-to-cell variability in that case is more complicated than in the steady-state case[17].

Another challenge in converting mRNA statistics to gene activity is the presence of multiple gene copies in the single cell. An *E. coli* cell growing in rich medium at 37 °C may contain up to eight copies of a chromosomal gene[33], with different cells in the population having different copy numbers. The observed number of mRNA molecules in a given cell reflects the combined stochastic activity of each of these gene copies. Neglecting such dosage effects will lead to distorted parameter estimation[9]. To avoid this, cells can be grown at a slow growth rate (>80 min generation time[34]). Under such conditions, a chromosomal gene will only replicate once in the cell cycle, and cells having one versus two gene copies can be discriminated on the basis of cell length[9].

MATERIALS

REAGENTS

▲ **CRITICAL** Use RNase- and DNase-free materials whenever possible.

- 6-Carboxytetramethylrhodamine, succinimidyl ester (6-TAMRA; Life Technologies/Invitrogen, cat. no. C6123)
- Sodium hydroxide (Fisher Scientific, cat. no. BP359-500)
- DMSO (Fisher Scientific, cat. no. BP231-100)
- Sodium bicarbonate (Fisher Scientific, cat. no. BP328-500)
- Sodium acetate, anhydrous (Fisher Scientific, cat. no. BP333-500)
- Glacial acetic acid (Fisher Scientific, cat. no. BP2401-500) **! CAUTION** Glacial acetic acid is extremely volatile and can cause severe burns on direct contact with skin or eyes, or if inhaled. Handle the compound with protective clothes under a fume hood.
- Ethanol (Decon Labs, cat. no. 2716) **! CAUTION** Ethanol is flammable.
- Tris-EDTA (TE, 1×; Fisher Scientific, cat. no. BP2473-100)
- FISH DNA probes modified with a 3′ amine group, or alternatively, already labeled with a fluorescent dye (Biosearch Technologies). To study the *lacZ* mRNA, we designed 72 antisense probes. See Reagent Setup for design details and sequences
- *E. coli* strains expressing the gene of interest, as well as a negative control strain where the gene of interest is deleted. For example, to study the expression of *lacZ* from the *lac* promoter (P_{lac}), we use the strain TK310 (Δ*cyaA* Δ*cpdA* Δ*lacY* (ref. 35)). As a negative control, we use the strain BW14894 (Δ*lacIZYA* (ref. 36))
- Bacterial growth medium: defined media are preferred over rich media (such as LB), because they typically provide more reproducible results. For example, to study the expression of *lacZ* from P_{lac}, we used a defined medium (M9CAgluc, Teknova, cat. no. M8010) composed of 1× M9 salts + 1% (wt/vol) glucose + 0.1% (wt/vol) casamino acids + 0.5 μg ml^{-1} thiamine + 0.2 mM magnesium sulfate + 0.1 mM calcium chloride
- Chemical inducers: for example, to induce P_{lac}, we use isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma-Aldrich, cat. no. 16758-1G) and adenosine 3′-5′-cyclic monophosphate (cAMP; Sigma-Aldrich, cat. no. A9501)
- PBS, RNase free (10×; Life Technologies/Ambion, cat. no. AM9625)
- SSC, RNase free (20×; Life Technologies/Ambion, cat. no. AM9763)
- Diethylpyrocarbonate (DEPC)-treated water (Life Technologies/Ambion, cat. no. AM9922)
- BSA, RNase and DNase free (50 mg ml^{-1}; Life Technologies/Ambion, cat. no. AM2616)
- Formamide, deionized, nuclease free (Life Technologies/Ambion, cat. no. AM9342) **! CAUTION** 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.
- *E. coli* tRNA (Sigma-Aldrich, cat. no. R4251-500UN)
- Dextran sulfate sodium salt (Sigma-Aldrich, cat. no. D8906-10G)
- Ribonucleoside-vanadyl complex (VRC, 200 mM; New England Biolabs, cat. no. S1402S)
- Formaldehyde (37% (vol/vol), Fisher Scientific, cat. no. BP531-500) **! CAUTION** Formaldehyde is highly toxic and a known carcinogen. Handle it under a fume hood while wearing a lab coat and protective gloves.
- 4′,6-Diamidino-2-phenylindole, hydrochloride (DAPI; Fisher Scientific, cat. no. PI-46190)
- Low-melt agarose, RNase and DNase free (Fisher Scientific, cat. no. BP160-100)

EQUIPMENT

- Microcentrifuge tubes (National Scientific: 1.5 ml, cat. no. CN1700-BP; 2 ml, cat. no. CN2000-BP)
- PCR tubes (200 μl; Eppendorf, cat. no. 951010006)
- Tabletop centrifuge (Eppendorf, cat. no. 5424)
- Vortex (Fisher Scientific, cat. no. 02-215-365)
- NanoDrop 2000 Spectrophotometer (Thermo Scientific)
- Aluminum foil (Reynolds wrap, heavy duty)
- Luer-Lok syringes (BD Biosciences: 10 ml, cat. no. 309604; 30 ml, cat. no. 309650)
- Syringe filters (0.22 μm; Millipore, cat. no. SLGP033RS)

- Kimwipes (VWR, cat. no. 82003-820)
- Parafilm M (VWR, cat. no. 52858-000)
- Falcon round-bottom polypropylene tubes (14 ml; BD Biosciences, cat. no. 352059)
- Orbital air shaker (Thermo Scientific, MaxQ4000, cat. no. SHKE4000). Alternatively, use an orbital water shaker (Thermo Scientific, MaxQ7000, cat. no. SHKE7000)
- Baffled shaker flasks (250 ml; Fisher Scientific, cat. no. 09-552-34)
- Motorized pipette filler (Fisherbrand, 03-692-164)
- Serological pipettes (Corning; 10 ml, cat. no. 4488; 50 ml, cat. no. 4490)
- Spectrophotometer (Bio-Rad, SmartSpec Plus spectrophotometer, cat. no. 170-2525)
- Spectrophotometer cuvettes (Bio-Rad, cat. no. 233-9955)
- Conical-bottom centrifuge polypropylene tubes (50 ml; Corning, cat. no. 430828)
- Centrifuge (Thermo Scientific, Sorvall Legend XTR, cat. no. 75004521)
- RNase-free pipette tips (Fisherbrand: 0.2–2.5 µl, cat. no. 02-707-442; 10–100 µl, cat. no. 02-707-431; 100–1250 µl, cat. no. 02-707-404)
- Nutating mixer (VWR, cat. no. 82007-202)
- Water bath (Fisher Scientific, Isotemp, cat. no. 15-462-20)
- Incubator (Fisher Scientific, Isotemp, cat. no. 11-690-637-D)
- Microscope slides (Fisherbrand, cat. no. 12-550-A3)
- Microscope cover glass (Fisherbrand: 24×50-1, cat. no. 12-544-E; 22×22-1, cat. no. 12-541-B)
- Kimax-35 glass media lab bottle (100 ml; VWR, cat. no. 16171-004)
- Plastic wrap (Saran)
- Razor blades (Garvey, no. 9, cat. no. 40475)
- Test weights (200 g; McMaster-Carr, cat. no. 1777T28)
- Optical table (TMC, breadboard, cat. no. 78-30551-01; four-post support, cat. no. 63-36186-02)
- Wide-field inverted fluorescence microscope (Nikon, Eclipse-T*i*)
- High-numerical-aperture (NA), oil-immersion objective (Nikon, ×100 Plan Apo 1.4 NA)
- Immersion oil A (Nikon, cat. no. MXA20233)
- Band-pass filters to separate fluorescence signals (for 6-TAMRA: Nikon, HC HISN Zero Shift, cat. no. 96365; for DAPI: Nikon, UV-2E/C, cat. no. 96310)
- Mercury lamp (Nikon, Intensilight C-HGFIE)
- High-sensitivity electron-multiplying charge-coupled device (EM-CCD) camera (Photometrics, Cascade II 1024)
- Motorized optical shutter (Sutter Instruments, SmartShutter)
- Motorized stage controller (PRIOR, H31 ProScan III)
- Universal specimen holder (Nikon, cat. no. H473XR)
- Microscope management software (Nikon, NIS-Elements)
- MATLAB (The MathWorks)
- Schnitzcells program (Elowitz laboratory[30], http://cell.caltech.edu/schnitzcells/)

REAGENT SETUP

Probe design and labeling Probe design is based on the protocol described in refs. 4,37. In brief, a set of 48–72 oligonucleotide probes is designed to bind to the target RNA. As guidelines for the design, we set the probes to be between 17 and 22 (typically 20) nt long, keeping an interprobe separation of at least two nucleotides and a GC content as close as possible to 45%. To design such a set, we use the Probe Designer algorithm developed by Arjun Raj (available at http://www.singlemoleculefish.com/). To study *lacZ* expression, we designed 72 probes spanning the whole coding sequence of the *lacZ* gene; their sequences, from 5′ to 3′, are in the table at right. Custom probes can be ordered from several providers. We typically use Biosearch Technologies (http://www.biosearchtech.com/). Each probe is ordered with a 3′ amine group, which allows covalent modification with NHS-ester derivatives of fluorescent dye molecules (for example, 6-carboxytetramethylrhodamine, succinimidyl ester, see Reagents). Alternatively, the probes can be ordered prelabeled. However, we have typically found in-house labeling to be cheaper (per experiment). We order 10 nmol per probe, purified using a reverse-phase cartridge. We order our oligos in 96-well plates, diluted in 100 µl of water, giving a probe concentration of ~100 µM. Upon arrival, we let the probes thaw and then centrifuge the 96-well plate at 12,000*g* for 2 min at 4 °C. We then transfer each oligo solution to a 1.5-ml tube. The oligo solutions can be stored at −20 °C for several years.

GTGAATCCGTAATCATGGTC	TCACGACGTTGTAAAACGAC
ATTAAGTTGGGTAACGCCAG	TATTACGCCAGCTGGCGAAA
ATTCAGGCTGCGCAACTGTT	AAACCAGGCAAAGCGCCATT
AGTATCGGCCTCAGGAAGAT	AACCGTGCATCTGCCAGTTT
TAGGTCACGTTGGTGTAGAT	AATGTGAGCGAGTAACAACC
GTAGCCAGCTTTCATCAACA	AATAATTCGCGTCTGGCCTT
AGATGAAACGCCGAGTTAAC	AATTCAGACGGCAAACGACT
TTTCTCCGGCGCGTAAAAAT	ATCTTCCAGATAACTGCCGT
AACGAGACGTCACGGAAAAT	GCTGATTTGTGTAGTCGGTT
TTAAAGCGAGTGGCAACATG	AACTGTTACCCGTAGGTAGT
ATAATTTCACCGCCGAAAGG	TTTCGACGTTCAGACGTAGT
ATAGAGATTCGGGATTTCGG	TTCTGCTTCAATCAGCGTGC
ACCATTTTCAATCCGCACCT	TTAACGCCTCGAATCAGCAA
ATGCAGAGGATGATGCTCGT	TCTGCTCATCCATGACCTGA
TTCATCAGCAGGATATCCTG	CACGGCGTTAAAGTTGTTCT
TGGTTCGGATAATGCGAACA	TTCATCCACCACATACAGGC
TGCCGTGGGTTTCAATATTG	ATCGGTCAGACGATTCATTG
TGATCACACTCGGGTGATTA	ATACAGCGCGTCGTGATTAG
GATCGACAGATTTGATCCAG	AAATAATATCGGTGGCCGTG
TTTGATGGACCATTTCGGCA	TATTCGCAAAGGATCAGCGG
AAGACTGTTACCCATCGCGT	TGCCAGTATTTAGCGAAACC
AAACGGGGATACTGACGAAA	TAATCAGCGACTGATCCACC
GGGTTGCCGTTTTCATCATA	TCGGCGTATCGCCAAAATCA
TTCATACAGAACTGGCGATC	TGGTGTTTTGCTTCCGTCAG
ACGGAACTGGAAAAACTGCT	TATTCGCTGGTCACTTCGAT
GTTATCGCTATGACGGAACA	TTTACCTTGTGGAGCGACAT
GTTCAGGCAGTTCAATCAAC	TTGCACTACGCGTACTGTGA
AGCGTCACACTGAGGTTTTC	ATTTCGCTGGTGGTCAGATG
ACCCAGCTCGATGCAAAAAT	CGGTTAAATTGCCAACGCTT
CTGTGAAAGAAAGCCTGACT	GGCGTCAGCAGTTGTTTTTT
TACGCCAATGTCGTTATCCA	TAAGGTTTTCCCCTGATGCT
ATCAATCCGGTAGGTTTTCC	GTAATCGCCATTTGACCACT
AGTTTTCTTGCGGCCCTAAT	ATGTCTGACAATGGCAGATC
ATAATTCAATTCGCGCGTCC	TGATGTTGAACTGGAAGTCG
TCAGTTGCTGTTGACTGTAG	ATTCAGCCATGTGCCTTCTT
AATCCCCATATGGAAACCGT	AGACCAACTGGTAATGGTAG

Sodium hydroxide (1 M) Dissolve 2 g of sodium hydroxide in 50 ml of distilled water. Sterilize the solution by passing it through a 0.22-µm syringe filter. The solution can be stored at room temperature (~22 °C) for up to a year. ! CAUTION Sodium hydroxide is corrosive. Handle it while wearing a lab coat and protective gloves.

Sodium bicarbonate (1 M, pH 8.5) Dissolve 4.2 g of sodium bicarbonate in 50 ml of distilled water. Adjust the pH to 8.5 with 1 M sodium hydroxide. Sterilize the solution by passing it through a 0.22-µm filter. ▲ CRITICAL Freshly prepare the solution before use. The FISH probe labeling reaction is extremely sensitive to pH, and sodium bicarbonate solutions change their pH upon long storage. ! CAUTION Sodium hydroxide is corrosive. Handle it while wearing a lab coat and protective gloves.

Sodium bicarbonate (0.1 M, pH 9.0) Dissolve 0.42 g of sodium bicarbonate in 50 ml of distilled water. Adjust the pH to 9.0 with 1 M sodium hydroxide. Sterilize the solution by passing it through a 0.22-µm filter. ▲ CRITICAL Freshly prepare the solution before use. The FISH probe labeling reaction is extremely sensitive to pH, and sodium bicarbonate solutions change their pH upon long storage. ! CAUTION Sodium hydroxide is corrosive. Handle it while wearing a lab coat and protective gloves.

Sodium acetate (3 M, pH 5.2) Dissolve 123 g of sodium acetate in 400 ml of distilled water. Adjust the pH to 5.2 with glacial acetic acid. Bring it to a final volume of 500 ml. Aliquot the solution into glass bottles (100 ml per bottle) and sterilize it by autoclaving. Aliquots can be stored at room temperature for up to 1 year.

IPTG (500 mM) Dissolve 0.595 g of IPTG in 4 ml of distilled water and adjust the volume to 5 ml. Sterilize the solution by passing it through a 0.22-µm filter. Aliquot the solution into 1.5-ml tubes (1 ml per tube). Aliquots can be stored at –20 °C for up to a year.

DAPI (10 mg ml^{-1}) Dissolve 10 mg of DAPI in 1 ml of deionized water. Aliquot the solution into 200-µl PCR tubes (100 µl per tube) and wrap the tubes in aluminum foil. Aliquots can be stored at –20 °C for up to a year.

Growth media To study the expression of *lacZ* from P_{lac}, we use M9CAgluc medium with IPTG and cAMP as inducers. First, add 115 mg of cAMP to 35 ml of M9CAgluc medium to make a 10 mM cAMP M9CAgluc solution. To prepare the high-expression medium, combine 30 ml of this solution with 60 µl of 500 mM IPTG (final concentrations: 10 mM cAMP and 1 mM IPTG). To make the low-expression medium, combine 30 ml of M9CAgluc, 90 µl of 10 mM cAMP M9CAgluc solution and 60 µl of 500 mM IPTG (final concentrations: 0.03 M cAMP and 1 mM IPTG). Sterilize both solutions by passing them through a 0.22-µm filter. ▲ CRITICAL Freshly prepare the solutions before use.

Ribonucleoside-vanadyl complex Before opening the bottle, reconstitute the solution by incubating it in a 65 °C water bath for 10 min. Aliquot the solution into 200-µl PCR tubes (100 µl per tube). The solutions can be stored at –20 °C for several years.

PBS, 1× Combine 900 µl of DEPC-treated water and 100 µl of 10× PBS, RNase-free in a polypropylene tube. Scale up the volumes as necessary.

PBS, 1×, 3.7% (vol/vol) formaldehyde Combine 800 µl of DEPC-treated water, 100 µl of 10× PBS and 100 µl of 37% (vol/vol) formaldehyde in a polypropylene tube. Scale up the volumes as necessary. ! CAUTION Formaldehyde is highly toxic and a known carcinogen. Handle it under a fume hood while wearing a lab coat and protective gloves, and dispose of it according to relevant safety regulations. ▲ CRITICAL Freshly prepare the solution before use.

Formamide After opening the bottle, transfer the solution into 2-ml tubes (~1.9 ml per tube, to keep each tube as air-free as possible) and wrap the caps with Parafilm. The aliquots can be stored at –20 °C for several years. ! CAUTION Formamide is highly toxic and a known teratogen. Handle it under a fume hood while wearing a lab coat and protective gloves.

Wash solution (40% (wt/vol) formamide, 2× SSC) In a polypropylene tube, combine 100 µl of 20× SSC, 353 µl of formamide and 547 µl of DEPC-treated water. Scale up the volumes as necessary. Wrap the cap with Parafilm and store the tube at 4 °C or on ice during the protocol. ! CAUTION Formamide is highly toxic and a known teratogen. Handle the compound under a fume hood while wearing a lab coat and protective gloves. ▲ CRITICAL Freshly prepare the solution before use.

Wash solution with DAPI (40% (wt/vol) formamide, 2× SSC, 10 µg ml^{-1} DAPI) In a polypropylene tube, combine 100 µl of 20× SSC, 353 µl of formamide, 547 µl of DEPC-treated water and 1 µl of 10 mg ml^{-1} DAPI. Wrap the cap with Parafilm and store the tube at 4 °C or on ice during the protocol. ! CAUTION Formamide is highly toxic and a known teratogen. Handle it under a fume hood while wearing a lab coat and protective gloves. ▲ CRITICAL Freshly prepare the solution before use.

Hybridization solution (40% (wt/vol) formamide, 2× SSC) This recipe is adapted from http://www.einstein.yu.edu/uploadedFiles/LABS/robert-singer-lab/mammalian_insitu.pdf; see also ref. 6. Add 5 ml of DEPC-treated 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 on an orbital shaker until the bubbles disappear (~30 min). Meanwhile, take the formamide out of the freezer and bring it to room temperature before opening. Add to the solution 3,530 µl of formamide, 10 mg of *E. coli* tRNA, 1 ml of 20× SSC, 100 µl of 200 mM VRC and 40 µl of 50 mg ml^{-1} BSA. Bring the final volume to 10 ml by adding DEPC-treated water, and nutate the solution until it is homogenous. Sterilize the solution by passing it through a 0.22-µm filter. Transfer the solution to 1.5-ml tubes (500 µl per tube). The solutions can be stored at –20 °C for up to a year. ! CAUTION Formamide is highly toxic and a known teratogen. Handle it under a fume hood while wearing a lab coat and protective gloves.

Agarose pads Wash six microscope slides with 100% ethanol and rinse them with distilled water. Dry the surfaces with a Kimwipe. Stack five slides on a leveled surface (**Fig. 2a**). Add 20 ml of 1× PBS and 0.3 g of low-melt agarose to a 100-ml Kimax-35 bottle. Dissolve the contents by heating the bottle in a microwave at low power for 5 min, swirling the solution every 1 min. Pour the molten agarose solution onto the slides (**Fig. 2b**). Cover the agarose with the remaining slide, placing a 200-g weight on top (**Fig. 2c**). Allow the agarose to solidify for 45 min at room temperature. Remove the four slides from the sides of the agarose pad, leaving the top and bottom slides for easy storage and handling (**Fig. 2d**). Remove the excess agarose from the slides with a razor blade (**Fig. 2e**). For use in imaging, carefully move the slides, exposing 1 cm of the agarose pad, and excise a 1 × 1-cm agar pad with a razor blade (**Fig. 2f**). The slide-encased agarose pads can be wrapped in plastic wrap and stored at 4 °C for up to 24 h. ▲ CRITICAL Use slow heating to help dissolve the agarose without evaporating too much water, which may cause the agar to be viscous and hard to handle. Bubbles in the agarose pad can be avoided by placing the top slide with one edge first, then laying the slide down to cover the liquid agarose.

EQUIPMENT SETUP

Microscopy setup For imaging, we use a conventional inverted epifluorescence microscope (Nikon, Eclipse T*i*) equipped with a cooled EM-CCD camera (Photometrics, Cascade II: 1024) and motorized stage control (Prior, Proscan III). A mercury lamp is used as the light source (Nikon, Intensilight C-HGFIE). A fast motorized optical shutter (Sutter Instruments, SmartShutter) is used to control the fluorescence illumination exposure time. Band-pass filter cubes (Nikon) are used for spectral separation. An ×100, NA 1.40, oil-immersion phase-contrast objective (Nikon, Plan Apo ×100/1.40 oil) is used with an additional ×2.5 lens in front of the camera. The specimen is mounted on a universal specimen holder. The microscope is installed on an optical table (TMC, breadboard and four-post support) to dampen mechanical vibrations. Microscope management software (Nikon, Elements) is used to control the microscopy setup.

Many alternatives exist to our setup above. When you are choosing a microscopy system for quantitative imaging, the most important feature to consider is photon collection efficiency. This can be achieved by combining a high-quantum-efficiency CCD camera, a strong light source and a high-NA (>1.3) objective. Alternative equipment that we have used successfully with this protocol include the following: an Eclipse TE2000-E microscope (Nikon), Cascade 512 and Evolve 512 cameras (Photometrics) and Metamorph software (Molecular Devices).

PROCEDURE

Labeling FISH probes ● TIMING overnight

▲ CRITICAL Use RNase- and DNase-free materials whenever possible.

1| Take equal volumes of each of the 100 µM oligo solutions and pool them in a 1.5-ml tube to give a final volume of 360 µl. Add 40 µl of 1 M sodium bicarbonate. Mix the contents thoroughly by pipetting. For example, to label our *lacZ* probe set, we take 5 µl of each of the 72 oligo solutions to obtain a final volume of 360 µl.

2| Weigh 1 mg of succinimidyl-ester-modified dye (for example, 6-TAMRA) in a 2-ml tube and dissolve it in 2.5 µl of DMSO. Add 25 µl of 0.1 M sodium bicarbonate and mix it thoroughly by pipetting.

▲ CRITICAL STEP Succinimidyl-ester dyes are highly unstable upon dilution in DMSO. Dissolve the dyes freshly before use and continue with the next step immediately. Wrap the tube in aluminum foil to protect the solution from light.

3| Add the oligo solution from Step 1 to the dye solution from Step 2. Mix the solutions thoroughly by pipetting. Wrap the tube in aluminum foil. Incubate the mixture in the dark overnight at 37 °C.

▲ CRITICAL STEP The optimal pH for the probe labeling reaction is in the range of 8.5–9.3. The use of a buffer with a pH outside this range may decrease the probe labeling efficiency (LE; see refs. 4,6 and manufacturer's instructions). If desired, a larger or smaller amount of probes can be labeled. In that case, scale the amount of reagents so that the final concentration of probes, dye, sodium bicarbonate and sodium acetate remains the same.

Ethanol precipitation ● TIMING 1–1.5 d

4| Add 47 µl of 3 M sodium acetate to the labeled oligo solution from Step 3. Mix the solution thoroughly by pipetting.

5| Add 1,180 µl of 100% ethanol. Mix the solution thoroughly by pipetting. Place the mixture at –80 °C for at least 3 h and up to overnight.

6| Centrifuge the tube at 15,000*g* for 30 min at room temperature in a tabletop centrifuge. Decant the supernatant. Remove the remaining liquid using a Kimwipe, while avoiding touching the pellet.

7| Dissolve the pellet in 45 µl of DEPC-treated water. Add 5 µl of 3 M sodium acetate. Mix the solutions thoroughly by pipetting.

8| Add 125 µl of 100% ethanol. Mix the contents of the tube thoroughly by pipetting. Place the mixture at –80 °C for at least 3 h and up to overnight.

9| Repeat Steps 6–8 once more.

10| Resuspend the pellet in a total of 250 µl of 1× TE to make the 10× probe stock solution. Transfer 50 µl of this solution to a 1.5-ml tube and add 450 µl of 1× TE to make the 1× probe stock solution. Transfer the 1× stock into 1.5-ml tubes (50 µl per tube), and wrap the 10× and 1× stocks in aluminum foil.

▲ CRITICAL STEP Preparing aliquots with a small volume (50 µl per tube) of the 1× stock helps avoid probe degradation caused by repeated freeze-thaw cycles. Keep the tubes on ice while using the stocks.

Measuring probe LE ● TIMING 30 min

11| The probe LE is defined as LE = [dye (µM)]/[probe (µM)]. With a spectrophotometer, measure [DNA (µg ml^{-1})] and [dye (µM)] for the 1× probe stock solution. Next, calculate [probe (µM)] using the formula

$$[\text{probe}\,(\mu M)] = (1{,}000 / MW_{DNA}) * [\text{DNA}\,(\mu g\ \mu l^{-1})]$$

where MW_{DNA} is the approximate molecular weight of a single-stranded DNA, given by

$$MW_{DNA} = \#\text{nucleotides} * 303.7\,(g\ mol^{-1})$$

To record these measurements, we use the NanoDrop 2000 under the 'Microarray' application, which allows the simultaneous measurement of [DNA (µg ml^{-1})] and [dye (µM)]. The DNA concentration of the 1× stock solution should be 10–16 µM.

We typically obtain probe LEs higher than 90%. Independent measurements of the probe LE using HPLC yield similar results, and thus we generally do not HPLC-purify our probes. However, if significantly lower probe LEs are obtained, HPLC purification is advisable[37,38].

? TROUBLESHOOTING

■ **PAUSE POINT** The 10× and 1× probe stock solutions can be stored at −20 °C for several years.

Growing cell cultures ● TIMING ~18–22 h

12| Add 2 ml of defined medium to two 14-ml polypropylene falcon tubes. Inoculate the cultures with single colonies of *E. coli* strains to be used in the experiment. Grow the cultures overnight (12–16 h) in an orbital shaker at 265 r.p.m. and at 37 °C.

13| The next morning, for each of the samples, add 30 ml of defined medium to a 250-ml baffled shaker flask. To study P_{lac}, we use at least three samples: the negative control strain BW14894 grown in M9CAgluc medium, the positive strain TK310 grown in low-expression medium and the positive strain TK310 grown in high-expression medium (see Reagent Setup for growth media).

14| Dilute the overnight cultures to 1:1,000 into the appropriate growth medium. Begin growing the overday cultures in an orbital shaker at 265 r.p.m. and at 37 °C.

▲ **CRITICAL STEP** It is advisable to measure the growth rate of the bacterial strains before performing the smFISH experiment. Depending on the growth rate, dilute the strains between 1:20,000 and 1:250 so that they reach an optical density at 600 nm (OD_{600}) of ~0.2 at approximately the same time. To study P_{lac}, we dilute the overnight cultures of BW14894 to 1:20,000 and TK310 to 1:250. The cultures take 5–6 h to reach an OD_{600} of ~0.2.

Fixation and permeabilization ● TIMING 2 h

15| About 1 h before collecting the cells, prepare an ice-water bath and prechill 50-ml centrifuge tubes for each of the cultures.

16| Fixation of cells can be performed by either collecting and resuspending them in formaldehyde ('standard method', option A below) or by adding formaldehyde directly to the cell culture ('direct method', option B below). We generally use the standard method, but the direct method may be used if one wishes to measure mRNA levels over multiple time points, or if one wishes to increase the throughput of the protocol.

(A) Standard method

(i) Measure the OD_{600} of the overday cultures in a spectrophotometer every ~1 h. When the OD_{600} is 0.2–0.4, collect a volume of culture having the same number of cells as 15 ml of culture at OD_{600} = 0.4 (this volume can be calculated as $V(\text{ml}) = 6/OD_{600}$). Transfer the collected culture to an ice-cold 50-ml centrifuge tube. Keep the tubes in the ice-water bath while collecting the cultures. Centrifuge the tubes at 4,500*g* for 5 min at 4 °C.

▲ **CRITICAL STEP** It is important that all samples have approximately the same number of cells at the time of collection. This is achieved by adjusting the volume of the collected culture to compensate for differences in OD_{600}.

(ii) Decant the supernatant and tap the inverted tubes on paper towels to remove the remaining liquid. Resuspend each pellet in 1 ml of ice-cold 1× PBS and 3.7% (vol/vol) formaldehyde. Transfer the mixture to 1.5-ml tubes. Mix gently for 30 min at room temperature using a nutator.

! CAUTION Formaldehyde is highly toxic and a known carcinogen. Handle it under a fume hood while wearing a lab coat and protective gloves, and dispose of it according to proper safety and environmental regulations.

(iii) Centrifuge the cells at 400*g* for 8 min at room temperature. Pipette out the supernatant and discard it.

(iv) Wash the cells twice in 1 ml of 1× PBS. Each time, add 1 ml of 1× PBS, and resuspend the cells by pipetting. Centrifuge the mixture at 600*g* for 3.5 min at room temperature, pipette out the supernatant and discard it.

(B) Direct method

(i) Measure the OD_{600} of the overday cultures in a spectrophotometer every ~1 h. When the OD_{600} is 0.2–0.4, add 37% (vol/vol) formaldehyde directly to the cell culture, to a final concentration of 3.7% (vol/vol) formaldehyde. Transfer a volume of culture having the same number of cells as 15 ml of culture at OD_{600} = 0.4 (this volume can be calculated as $V(\text{ml}) = 6/OD_{600}$) to a 50-ml polypropylene tube, and incubate it for 30 min at room temperature using a nutator.

! CAUTION Formaldehyde is highly toxic and a known carcinogen. Handle it under a fume hood while wearing a lab coat and protective gloves, and dispose of it according to proper safety and environmental regulations.

(ii) Centrifuge the cells at 400*g* for 8 min at room temperature. Pipette out the supernatant and discard it.
▲ **CRITICAL STEP** The pellet may be very loose. Be careful when pipetting out the supernatant.
(iii) Resuspend each pellet in 1 ml of 1× PBS. Transfer the mixture to 1.5-ml tubes. Centrifuge the mixture at 600*g* for 3.5 min at room temperature. Pipette out the supernatant and discard it.
(iv) Add 1 ml of 1× PBS and resuspend it by pipetting. Centrifuge the mixture at 600*g* for 3.5 min at room temperature. Pipette out the supernatant and discard it.

17| *Permeabilize the cells*. Resuspend the pellet in 300 µl of DEPC-treated water. Add 700 µl of 100% ethanol and mix it thoroughly by pipetting. Mix the cells gently for 1 h at room temperature by using a nutator.
▲ **CRITICAL STEP** Dissolving the pellet in water before adding ethanol prevents the formation of cell aggregates.
■ **PAUSE POINT** After mixing the cells at room temperature for 1 h, the cells can be kept in 70% (vol/vol) ethanol at 4 °C for up to 7 d without affecting the results.

18| While the cells are being permeabilized in ethanol, prepare a 40% wash solution. Keep this solution on ice.

Hybridization ● TIMING overnight
19| Centrifuge the cells at 600*g* for 7 min at room temperature. Carefully pipette out the supernatant and discard it.
▲ **CRITICAL STEP** The pellet may be very loose, or may be spread over the tube wall. Be careful when you are pipetting out the supernatant.

20| Resuspend the pellet in 1 ml of 40% wash solution. Mix the contents gently for 5 min at room temperature using a nutator.

21| In a new 1.5-ml tube, add 50 µl per sample of 40% hybridization solution. Add 1× probe stock solution to a final concentration of 1 µM. For example, if the concentration of the 1× stock is 15 µM, add 3.6 µl per sample. Mix the contents thoroughly by pipetting, avoiding bubbles.
▲ **CRITICAL STEP** For multicolor smFISH, add 1× stock solutions of each probe set to the hybridization solution, so that the final concentration of each probe set is 1 µM.

22| Centrifuge the cells at 600*g* for 7 min at room temperature. Pipette out the supernatant and discard it.

23| Add 50 µl of the hybridization solution with probes (Step 21) to the cell pellet. Resuspend the cells thoroughly by pipetting, avoiding bubbles. Incubate the mixture overnight (~14 h) at 30 °C.
▲ **CRITICAL STEP** The formamide concentration of the hybridization and wash solutions is the main parameter that controls the stringency of probe binding. Low formamide concentration (low stringency) favors nonspecific binding of probes. Increasing the formamide concentration (high stringency) reduces the nonspecific binding, but eventually also reduces the binding of probes to the target mRNA. Our protocol has been optimized for oligos that are 20 nt long with a GC content close to 45%. If the probe set departs from that standard, adjust the stringency of the hybridization and wash solutions by changing the concentration of formamide.
■ **PAUSE POINT** Cells in the hybridization solution can be stored at 4 °C for months, and the experiment can be resumed from this point without a visible decrease of signal quality.

Washing ● TIMING 2 h
24| Transfer 10 µl of the samples in hybridization reaction to a 1.5-ml tube. In the case that additional imaging sessions are needed, resume the protocol from this step using 10 µl of the remaining hybridization reaction.

25| Add 200 µl of 40% wash solution to the tube. Mix the contents thoroughly by pipetting. Centrifuge the tube at 600*g* for 3.5 min at room temperature. Pipette out the supernatant and discard it.

26| Add 200 µl of 40% wash solution. Mix the tube thoroughly by pipetting. Incubate the mixture for 30 min at 30 °C.

27| Mix the tube thoroughly by pipetting. Centrifuge the tube at 600*g* for 3.5 min at room temperature. Pipette out the supernatant and discard it.

28| Repeat Steps 26 and 27 once more. While the cells are being washed, prepare the agarose gel for imaging (see Reagent Setup), turn on the microscope and prepare the 40% wash solution with 10 μg ml^{-1} DAPI.

29| Add to the samples 200 μl of 40% wash solution with 10 μg ml^{-1} DAPI. Mix the tube thoroughly by pipetting. Incubate the tube for 30 min at 30 °C.

30| Mix the contents of the tube thoroughly by pipetting. Centrifuge the tube at 600*g* for 3.5 min at room temperature. Pipette out the supernatant and discard it.

31| Resuspend the cells in 10 μl of 2× SSC.
▲ **CRITICAL STEP** Pipette the cell suspension up and down multiple times to separate any cell aggregates before assembling the slides for imaging.

Microscopy setup and recording ● TIMING 2–4 h
32| *Assemble the slides for imaging*. For each sample to be imaged, take a 24 × 50-mm coverslip and pipette 2 μl of the cell suspension onto the center of the coverslip (**Fig. 2g**). Cut a 1 × 1-cm agarose pad with a razor blade (**Fig. 2f**). Lift the agarose pad from one corner with the blade and lay the pad slowly on top of the cell suspension droplet (**Fig. 2h**). Cover the pad with a 22 × 22-mm coverslip (**Fig. 2i**).
▲ **CRITICAL STEP** Be sure to use coverslips of thickness no. 1. Other commonly used coverslips (e.g., thickness no. 1.5) show a higher fluorescent background. We note that other smFISH protocols call for adding antifading reagents to the imaged sample[4,6].

33| *Set the imaging parameters*. To characterize the optimal imaging conditions for your experimental setup, first image the sample with the highest expression level. Find the best focal plane (*z*-position) in the phase contrast channel, and acquire images from the fluorescence channel. Repeat this procedure for different exposure times and stage positions (fields of view), recording the maximum pixel value of the fluorescent foci. We have found that a good rule is to choose exposure times that produce foci pixel values no higher than 60% of the maximum pixel value of the camera (maximum value of 65,535 for a 16-bit camera). For our imaging setup, exposures in the range of 250–500 ms fulfill this criterion. If the signal is still low, we also increase the electromultiplier gain of the CCD camera, as longer exposure times may lead to increased photobleaching. In addition, check that the chosen conditions allow the visualization of dim foci (coming from a single probe or a small set of overlapping probes) in the negative control sample. Repeat the same procedure for the DAPI channel.
▲ **CRITICAL STEP** For a multicolor smFISH experiment, the filters used have to be optimized to minimize the cross talk between channels. To check for the presence of cross talk, image single-color labeled samples under all the different filter sets.
? TROUBLESHOOTING

34| *Acquire the images at different focal planes (z-slices) for all channels* (**Fig. 1a**). We typically image the cells in nine successive *z*-slices at 200-nm spacing. Repeat the procedure for different stage positions (fields of view) either manually or by using an automatic acquisition mode if available. Acquire enough images so that ~1,000 cells are imaged per sample, typically 10–40 image positions.
▲ **CRITICAL STEP** A systematic scanning of the slide prevents imaging areas that have been illuminated previously. Check that the illuminated area is only slightly bigger than the field of view by changing the aperture of the fluorescent light diaphragm. Avoid areas where the cells are too dense, because cell recognition routines work better on images in which the cells are not in close contact.
? TROUBLESHOOTING

Data analysis ● TIMING 2–3 d
35| *Convert the data stacks to TIFF format*. Microscope controller software such as Nikon Elements or Metamorph save each field of view as a multidimensional data stack (.nd or .nd2 format). Convert these image stacks into standalone .tif images by using the built-in utilities inside the microscope controller software.

36| *Perform automatic cell segmentation*. We have used both Schnitzcells[30] and MicrobeTracker[39] software, but any program that generates cell segmentation masks (label matrix, readable by MATLAB, in which non-cell pixels have a value of zero and cell pixels have integer values corresponding to the cell identification number (**Fig. 1b**)) from phase-contrast images could be used.

37| *Perform automatic spot recognition using the Spätzcells program (***Fig. 1b***).* We developed Spätzcells to identify and measure the properties of fluorescent foci (spots) across multiple focal planes in image stacks. A copy of the program and the accompanying documentation are available upon request. The algorithm works as follows: Spätzcells first identifies 2D local maxima of fluorescence intensity, with height above a predefined 'spot detection threshold'. These maxima are then classified as spots only if they appear in multiple adjacent image planes (z positions). Finally, for each spot, the fluorescence intensity profile (at the focal plane where the spot is in focus) is fitted to a 2D Gaussian function, and features such as the position, peak height (amplitude of the Gaussian fit) and the integrated fluorescence intensity are recorded. In the case that other spots are present in the vicinity of the spot being fitted, a 2D multi-Gaussian fit is performed.
▲ **CRITICAL STEP** To accurately characterize mRNA spots, the spot detection threshold should be set low enough such that some false-positive spots are recognized in the negative control sample (~1 spots per cell) and all spots are recognized in the positive sample.
? TROUBLESHOOTING

38| *Select a 'false-positive threshold' to discard the spots resulting from nonspecific binding of probes.* The low spot detection threshold used to recognize spots in the previous step ensures that all genuine spots (i.e., spots corresponding to target mRNA) are recognized, at the price of increasing the number of false positives. To discard such false positives, compare the peak height distributions of the spots in the negative control sample to the ones in the low-expression control sample. Select a 'false-positive threshold' in peak height that separates the population of false positives from the population of genuine spots in the low-expression control sample (**Fig. 1c**). Spots with peak heights lower than this false-positive threshold are discarded from the subsequent analysis of all samples.

39| *Define the fluorescence intensity of a single mRNA.* After discarding the false positives, use the remaining spots in the low-expression control sample to construct a spot intensity histogram. This histogram should show a predominant species, corresponding to a single mRNA molecule (**Fig. 1d**). Use MATLAB, or another data analysis software, to fit the complete histogram to a sum of Gaussians with increasing peak positions and decreasing peak heights, corresponding to one, two, three and so on mRNA molecules per spot. Each Gaussian in this sum has a mean that is an integer multiple of the first Gaussian and a variance that scales with the mean, reflecting the statistical independence of labeling and detecting individual mRNAs[3]. Estimate the single-mRNA intensity as the mean of the first Gaussian.

40| *Convert the total fluorescence of the spots inside each cell into the number of mRNA molecules in the cell.* Sum the spot intensities from all the spots in a cell and divide this number by the intensity of a single mRNA molecule. Round the value to the closest integer.

41| *Use the population statistics to estimate transcription kinetics parameters.* We model transcription as a two-state process: the gene switches on and off, producing multiple mRNAs (a 'burst') during the 'on' period. Use MATLAB, or another data analysis software, to fit the histogram of mRNA copy numbers per cell to a negative binomial distribution (predicted by the two-state model[40]) (**Fig. 1e**):

$$P(n) = \binom{n+r-1}{n} p^r (1-p)^n$$

Where $P(n)$ is the probability of observing n mRNAs in a cell; r and p are fitting parameters. The values of r and p can be used to estimate the frequency, f, and size, b, of transcription bursts as follows: $f = r/\tau_{RNA}$, where τ_{mRNA} is the mRNA lifetime (measured separately using standard methods[8,9]), and $b = (1-p)/p$. An alternative method for estimating f and b is by using the relations $b = \sigma^2/\langle n\rangle$ and $f = \langle n\rangle^2/((\sigma^2-\langle n\rangle))\ \tau_{RNA}$, where $\langle n\rangle$ and σ^2 are the mean and variance, respectively, of the mRNA copy number in the sample. The two procedures typically yield very similar results[9] (for more details on these mathematical relations, see refs. 8,9).
▲ **CRITICAL STEP** The use of a negative binomial distribution to fit the mRNA copy-number histogram relies on assuming that the 'on' duration is much shorter than the mRNA lifetime. A more general expression for the mRNA copy-number distribution under the two-state model has been derived analytically[2,17]. However, for the majority of cases we have studied, we find that a negative binomial distribution fits the data very well[9].

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
11	Low probe labeling efficiency	Low dye/probe ratio	Increase the amount of dye used in the probe labeling reaction
33	Agarose pad appears inhomogeneous	Incomplete melting or excessive evaporation of agarose solution before casting	Melt agarose in a microwave using a low power setting. Check agarose solution every 1 min. When the agarose solution reaches boiling temperature, stop heating to avoid excessive evaporation. Swirl and verify that the solution is completely clear. If unmelted agarose is visible, boil the solution once more. Pour agarose solution into its cast immediately after boiling
		Agarose pad is damaged	Manipulate agarose pads with a razor blade, touching only the corner of the pad
	Cells move around or do not lie completely in the image plane	Agarose is too moist	Allow evaporation of agarose pad inside its cast for 1 h after casting. Alternatively, assemble coverslips and wait 15 min before imaging
	FISH signal in negative sample is high	Formamide concentration of hybridization and washing solution is too low for the probe set	Increase formamide concentration of hybridization and washing solutions (up to 50% (wt/vol) formamide). Increase the number and duration of washing steps (up to four washing steps of 1.5 h each)
		Free fluorescent dye in probe solution	Purify probes with additional rounds of ethanol precipitation
	FISH signal in positive sample is low (similar to signal in negative sample)	Formamide concentration of hybridization and washing solution is too high for the probe set	Scan a range of formamide concentrations (10–40% (wt/vol)) in increments of 10%
		Probe design is not optimal	Recheck that probes are antisense. Redesign probes with GC content close to 45%. Increase probe length up to 22 nt (refs. 4,38)
		Incomplete permeabilization	Increase incubation time in 70% (vol/vol) ethanol for up to 1 week at 4 °C
34	Nonuniform spot fluorescence in different areas of the slide	Images are taken over an already bleached area	Scan the slide in a systematic manner. Check that the illuminated area is only slightly bigger than the field of view by adjusting the field diaphragm
	Fluorescence signal bleaches	Sample is overilluminated	Decrease light intensity or exposure time
37	Spot recognition software takes too long to run	Spot detection threshold in Spätzcells is set too low. Area for spot fitting is too big. Computer is not powerful enough	Increase the spot detection threshold and decrease the fitting area for spots in Spätzcells. Use a computer with at least 4 GB of RAM and >2 GHz of CPU speed

● TIMING

Steps 1–3, labeling FISH probes: overnight
Steps 4–10, ethanol precipitation: 1–1.5 d
Step 11, measuring probe LE: 30 min
Steps 12–14, growing cell cultures: ~18–22 h

Steps 15–18, fixation and permeabilization: 2 h
Steps 19–23, hybridization: overnight
Steps 24–31, washing: 2 h
Steps 32–34, microscopy setup and recording: 2–4 h
Steps 35–41, data analysis: 2–3 d (depends on the user's proficiency)

ANTICIPATED RESULTS

smFISH followed by automated image analysis allows the quantification of mRNA copy numbers from a gene of interest in individual *E. coli* cells. **Figure 1** shows the experimental workflow and representative data sets for the case of the endogenous *lacZ* gene in *E. coli*. Cell cultures of a strain lacking the *lac* operon (BW14894, negative control[36]) and strains expressing *lacZ* at low and high levels (TK310 (ref. 35) grown with varying amounts of IPTG and cAMP) were fixed, permeabilized, hybridized with 6-TAMRA–labeled probes, washed and imaged according to our protocol described above. When the fluorescence images are inspected closely, it can be seen that each sample contains spots of distinct brightness and size (**Fig. 3**). The negative control sample (**Fig. 3**, left columns) shows dim 'background' spots, corresponding to probes nonspecifically bound in the cell. The low-expression control sample (**Fig. 3**, middle columns) shows brighter, well-defined spots that correspond to single mRNAs, as well as background spots. The high-expression sample (**Fig. 3**, right columns) shows spots that are brighter and more variable than those visible in the low-expression control sample, reflecting multiple overlapping mRNA molecules. In the high-expression sample, background spots are typically not visible.

The properties of individual fluorescent spots are estimated by using the Spätzcells program (**Fig. 1b**, right). We have found that the most robust feature for differentiating bona fide mRNA spots from false positives is their peak height (amplitude of the Gaussian fitted by Spätzcells). A low-expression control sample typically shows a peak-height distribution with two well-separated spot populations (**Fig. 1c**, red line). The values in the low-intensity population correspond closely to values found in the negative control sample (**Fig. 1c**, black line). A false-positive threshold in spot peak height is then chosen so as to separate the two spot populations in the low-expression control sample. Thereafter, in every sample examined, spots with peak heights lower than this threshold (**Fig. 1c**, shaded region) are discarded from the analysis.

After discarding false-positive spots, the spot intensity (intensity integrated over the area of the Gaussian fitted by Spätzcells) corresponding to a single mRNA molecule is identified by examining the histogram of spot intensities in a low-expression control sample, where individual mRNAs are spatially separable (**Fig. 1d**). We have found that the spot intensity histogram for samples with a mean of ≲3 mRNA per cell shows a well-defined single mRNA peak, which can be successfully used for calibration purposes. In some cases, the peaks corresponding to two, three and so on mRNAs can also be seen in the histogram (**Fig. 1d**).

The measured single-mRNA intensity value is next used to convert the total intensity of all spots in a given cell to the number of target mRNA molecules in that cell. By measuring mRNA numbers in >1,000 cells, the population mean and variance are estimated (**Fig. 1e**). The copy-number histogram is fitted to a simple model of transcription kinetics[8,9]. The parameters of the fit are used to calculate the frequency and size of transcription bursts (**Fig. 1e**). By using this analysis, the negative control sample typically shows a mean level and standard deviation of less than one mRNA. The estimated burst size and frequency for that sample (here, 0.4 mRNA and 0.1 min^{-1}, respectively) are indicative of the accuracy with which those parameters can be calculated in higher-expression samples. For the experiment depicted in **Figure 1e**, the low-expression control sample has ~3 mRNAs per cell (with burst size and frequency of 1.9 ± 0.1 mRNA and 0.7 ± 0.2 min^{-1}, respectively), whereas the same strain in high-expression conditions shows a mean level of ~50 mRNAs per cell (with burst size and frequency of 18.8 ± 1.3 mRNA and 1.3 ± 0.2 min^{-1}, respectively).

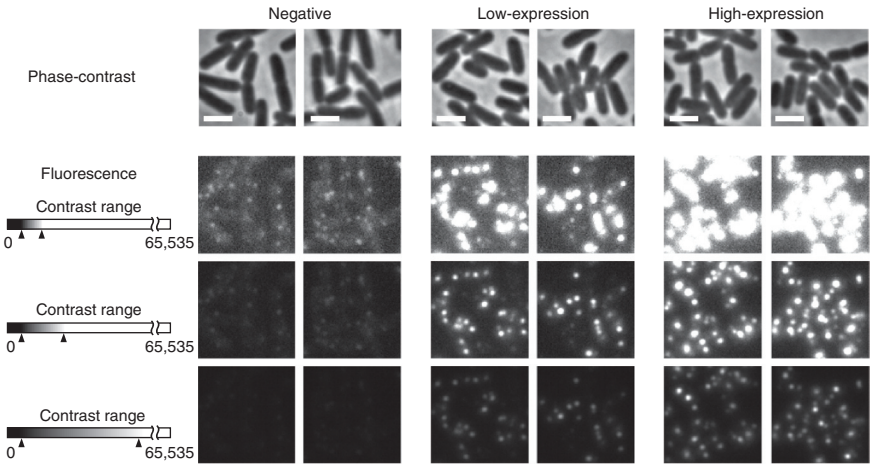

Figure 3 | Different smFISH samples contain spots of distinct brightness and size. Fluorescence channel images from three experimental samples (from left to right: negative control sample, low-expression control sample, high expression sample) are displayed at varying contrast levels (rows 2–4). The phase-contrast and fluorescence images were taken at a single focal plane (*z* position). Scale bars, 2 μm. See ANTICIPATED RESULTS for a discussion of the observed features.

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Corrigendum: Measuring mRNA copy number in individual *Escherichia coli* cells using single-molecule fluorescent *in situ* hybridization

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In the version of this article initially published, a component (40 µl of 50 mg ml^{-1} BSA) was erroneously omitted from the 'Hybridization solution' recipe in the Reagent Setup section. The error has been corrected in the HTML and PDF versions of the article.