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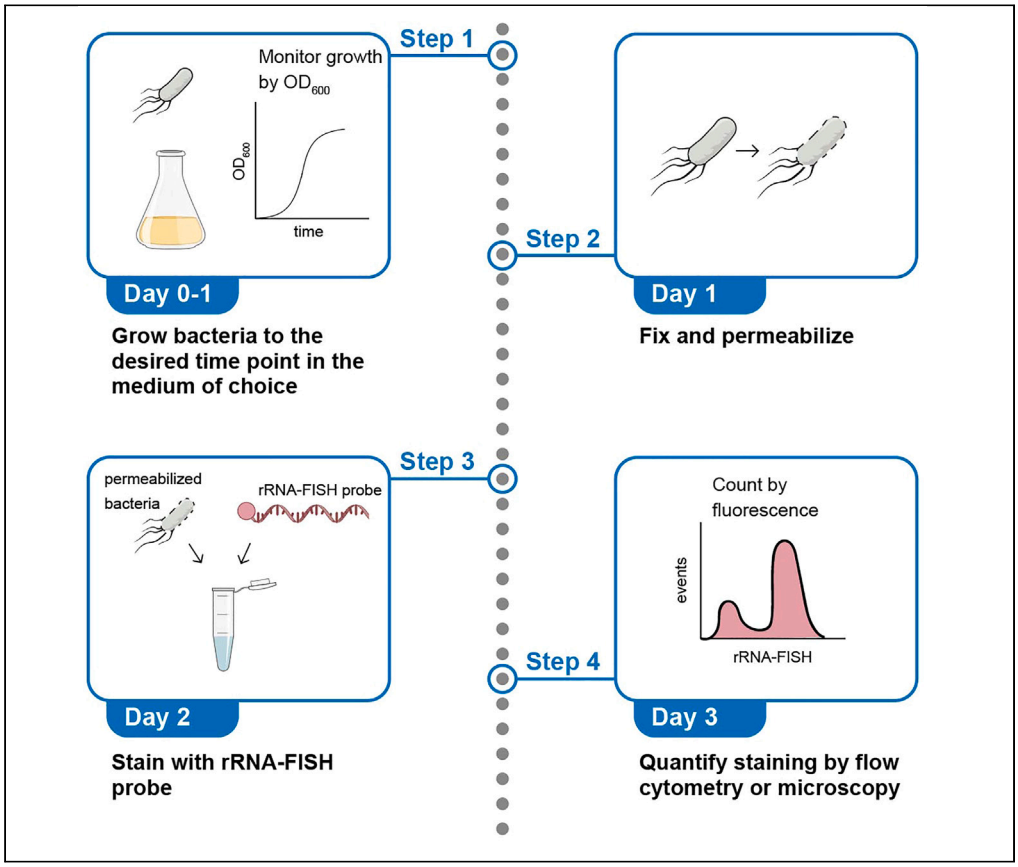
Protocol for comparing ribosomal levels in single bacterial cells at different growth stages using rRNA-FISH



Ribosome quantification in single cells is typically achieved through fluorescence tagging of ribosomal proteins. Here, we present a protocol for comparing ribosomal levels in bacteria at different growth stages using fluorescence *in situ* hybridization of rRNA (rRNA-FISH), eliminating the need for genetic engineering of the strain of interest. We detail the steps for preparing bacterial samples, staining with fluorescent probes, and acquiring data using flow cytometry and microscopy. Furthermore, we provide guidelines on controlling for proper labeling through signal localization analysis.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Bacterial samples are grown, harvested, fixed, and permeabilized

Cells are hybridized with rRNA-FISH probes targeting 16S rRNA

Samples are analyzed using flow cytometry and microscopy

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Protocol for comparing ribosomal levels in single bacterial cells at different growth stages using rRNA-FISH

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SUMMARY

Ribosome quantification in single cells is typically achieved through fluorescence tagging of ribosomal proteins. Here, we present a protocol for comparing ribosomal levels in bacteria at different growth stages using fluorescence *in situ* hybridization of rRNA (rRNA-FISH), eliminating the need for genetic engineering of the strain of interest. We detail the steps for preparing bacterial samples, staining with fluorescent probes, and acquiring data using flow cytometry and microscopy. Furthermore, we provide guidelines on controlling for proper labeling through signal localization analysis.

For complete details on the use and execution of this protocol, please refer to Ciolli Mattioli et al.[1]

BEFORE YOU BEGIN

The protocol describes how to fluorescently label 16S and 23S rRNAs to quantify ribosomes by flow cytometry analysis and microscopy in *Salmonella enterica* serovar Typhimurium (*S*.Tm) at several growth phases. We have also used this protocol for the Gram-negative *S.* typhi, *E. coli*, and for the Gram-positive *S. aureus* without modifications. If working with different bacteria, please see the limitations section for information about probe coverage in different microorganisms.

Institutional permissions

According to biosafety guidelines, *S*.Tm and *S. aureus* are classified as biosafety level 2 pathogens, and S. typhi as biosafety level 3 pathogen. Experiments using these pathogens should be approved by the institutional biosafety committee and conducted according to the institutional biosafety committee guidance.

Reagent preparation

Timing: 5 h

Stock solutions should be prepared ahead of use.

1. Reconstitute the 200 mM vanadyl ribonucleoside complex to form a green-black clear solution by incubating the sealed vial at 65°C in a water bath for 10 min.
   a. Aliquot the solution into PCR vials (100 µL per vial).
   b. Store the solution at −20°C.

2. Reconstitute the *E. coli* tRNA powder in deionized, nuclease-free water to a concentration of 20 mg/mL.
   a. Aliquot it into 500 μL vials.
   b. Store at −20°C.
3. Reconstitute DAPI powder in DMSO to a concentration of 10 mg/mL (1000x stock).
   a. Aliquots in 50 μL in amber vials.
   b. Store at −20°C.
4. Reconstitute glucose oxidase powder in 50 mM sodium acetate pH 5.2 to a concentration of 3.7 mg/mL, incubating at 37°C for 1 h.
   a. Aliquot the solution into 5 μL aliquots in PCR vials.
   b. Store at −20°C.
5. rRNA-FISH probes are ordered from Integrated DNA Technologies already conjugated with a fluorophore (Atto647 for 16S, Cy3 for 23S, Atto647 and Cy3 for the negative control), and with RNase-Free HPLC purification. They are shipped dehydrated.
   a. Upon arrival, reconstitute in 1x Tris-EDTA (10 mM Tris, 1 mM EDTA) pH 8 at a stock concentration of 100 μM.
   b. Prepare 10 μL aliquots, and store at −20°C.

⚠ CRITICAL: All consumables and reagents must be Nuclease-Free.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial strains		
*S.*Tm 14028S WT	ATCC	https://www.atcc.org/products/14028
E. coli K12-MG1655	Bacteriology and Genomic Repository, Weizmann Institute	
Chemicals, peptides, and recombinant proteins		
Pierce 16% formaldehyde (w/v), methanol-free	Thermo Fisher Scientific	Cat#28906
Dextran sulfate	Sigma-Aldrich	Cat#D8906-5G
Formamide	Roche	Cat#11814320001
E. coli tRNA	Sigma-Aldrich	Cat#R4251
20x SSC	Thermo Fisher Scientific	Cat#AM9765
UltraPure BSA	Thermo Fisher Scientific	Cat#AM2616
Ribonucleoside vanadyl complex	NEB	Cat#S1402S
DAPI	Roche	Cat#10236276001
Tris (1 M), pH 8.0, RNase-free	Thermo Fisher Scientific	Cat#AM9856
100x Tris-EDTA buffer solution	Sigma-Aldrich	Cat#T9285
Nuclease-free water	Thermo Fisher Scientific	Cat#AM9930
Glucose oxidase, type VII from *Aspergillus niger*	Sigma-Aldrich	Cat#G2133
Catalase from *Aspergillus niger*	Sigma-Aldrich	Cat#C3515
Phosphate-buffered saline (10X) pH 7.4, RNase-free	Thermo Fisher Scientific	Cat#AM9625
D-(+)-glucose	Sigma-Aldrich	Cat#G8270
Dimethyl sulfoxide	Sigma-Aldrich	Cat#D8418
LB broth (Miller)	Sigma-Aldrich	Cat#L3522
DPBS, no calcium, no magnesium	Biological Industries	Cat#02-023-1A
Oligonucleotides		
rRNA-FISH 16S: /5ATTO647/CTGCCTCCCGTAGGAGTC	Integrated DNA Technologies	
rRNA-FISH Atto647 negative control probe: /5ATTO647/ AACCCCTGATTGTATCCGCA	Integrated DNA Technologies	
rRNA-FISH 23S: /5Cy3/GCATCTTCACAGCGAGTTC	Integrated DNA Technologies	
rRNA-FISH Cy3 negative control probe: /5Cy3/ AACCCCTGATTGTATCCGCA	Integrated DNA Technologies	

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
rRNA-FISH pre16S probe: /5Cy3/TGCCCACACAGATTGTCTG ATAAATTGTTAAAGAGCAGTGC	Integrated DNA Technologies	
Software and algorithms		
MATLAB R2021b	MathWorks, Inc.	https://www.mathworks.com/products/matlab.html
ImageJ	Schneider et al.[2]	https://imagej.nih.gov/ij/index.html
Other		
Polysine slides	Epredia	Cat#P4981-001
Cover glass 24 × 60 mm thick #1	Bar Naor	Cat#BN1052441C
Round-bottom polystyrene test tubes	Falcon	Cat#352054
Cuvettes	Sarstedt	Cat#67.742
BD FACSAria III	BD Biosciences	
Eclipse Ti-E inverted microscope	Nikon	
Ultrospec 10 Cell density meter	Harvard Biochrom	
Excella E24 Incubator shaker	Eppendorf	

MATERIALS AND EQUIPMENT

Hybridization buffer		
Reagent	Final concentration	Amount
Dextran sulfate	10%	1 g
Formamide	25%	2.5 mL
E. coli tRNA 20 mg/mL	1 mg/mL	500 µL
20x SSC	2x	1 mL
Ribonucleoside Vanadyl Complex 200 mM	2 mM	100 µL
BSA 5%	0.02%	40 µL
Nuclease-Free H_2O	N/A	4.86 mL
Total	**N/A**	**10 mL**

Note: Prepare the hybridization buffer by first dissolving 1 g of dextran sulfate in 4.86 mL of Nuclease-Free water, agitating it for 30 minutes at 20°C–25°C on a roller. Upon solubilization of dextran sulfate, proceed by adding the remaining components. Aliquot into amber vials (500 µL per vial = 10 reactions per vial). Storage condition: −20°C, maximum storage: 1 year.

⚠ CRITICAL: Formamide is toxic and teratogenic. Handle it in a fume hood while wearing protective gloves and a lab coat, and dispose of it according to proper safety and environmental regulations.

Wash buffer		
Reagent	Final concentration	Amount
SSC 20x	2x	1 mL
Formamide	25%	2.5 mL
Nuclease-Free H_2O	N/A	6.5 mL
Total	**N/A**	**10 mL**

Note: Prepare without adding formamide, storage condition: 4°C, maximum storage: 1 year. Upon usage, prepare an aliquot according to the sample number by adding formamide (calculate 1 mL per sample). I.e., to prepare 1 mL of wash buffer, add 250 µL of formamide to 750 µL of wash buffer w/o formamide.

⚠ CRITICAL: Formamide is toxic and teratogenic. Handle it in a fume hood while wearing protective gloves and a lab coat, and dispose of it according to proper safety and environmental regulations.

Wash buffer with DAPI		
Reagent	Final concentration	Amount
SSC 20x	2x	1 mL
Formamide	25%	2.5 mL
DAPI 10 mg/mL	10 μg/mL	10 μL
Nuclease-Free H_2O	N/A	6.5 mL
Total	**N/A**	**10 mL**

Note: prepare fresh from the wash buffer (calculate 200 μL per sample).

⚠ CRITICAL: Formamide is toxic and teratogenic. Handle it in a fume hood while wearing protective gloves and a lab coat, and dispose of it according to proper safety and environmental regulations.

GLOX anti-fade buffer w/o enzymes		
Reagent	Final concentration	Amount
Tris pH 8 1 M	10 mM	10 μL
SSC 20x	2x	100 μL
Glucose 10%	0.4%	40 μL
Nuclease-Free H_2O	N/A	0.85 mL
Total	**N/A**	**1 mL**

Note: Aliquot in 100 μl units, storage condition: −20°C, maximum storage: 1 year.

Fixation solution		
Reagent	Final concentration	Amount
16% formaldehyde	3.7%	2.31 mL
10x PBS	1x	1 mL
Nuclease-Free H_2O	N/A	6.69 mL
Total	**N/A**	**10 mL**

Note: Adjust the volumes according to the number of samples processed (calculate 200 μL per sample). Storage condition: 4°C, maximum storage: 1 week.

⚠ CRITICAL: 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.

STEP-BY-STEP METHOD DETAILS

Cell starter – Day 0

Timing: 16–20 h

In this section, a bacterial starter is prepared.

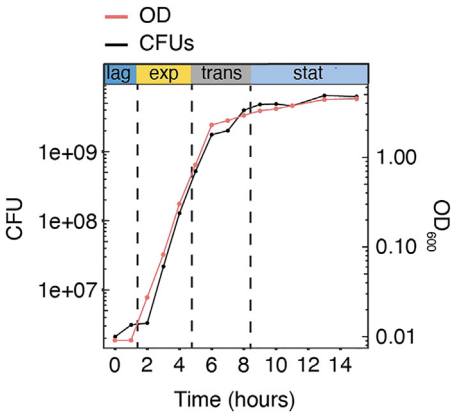

Figure 1. Growth curve of *S.*Tm measured by OD_{600} (in light red) and Colony Forming Units (CFUs) (in black)

Estimated growth phases are indicated on the top (lag; exp, exponential; trans, transition; stat, stationary). The gray area corresponds to the transition from exponential to stationary phase. This data is presented in Figure 1 in Ciolli Mattioli et al., 2023.[1]

1. Inoculate a bacterial colony from a plate into 5 mL of liquid rich medium compatible with the bacteria of choice and grow it overnight for 16–20 h at 37°C with shaking at 150–200 rpm.

   Note: see the limitations section for details about bacteria compatible with this protocol. If working with bacteria other than *S.*Tm or *E.coli*, we recommend preparing the positive controls with either of these bacterial species, grown in LB.

Cell preparation – Day 1

Timing: Variable, depending on the medium used and the growth phase of interest

In this section, the bacterial samples are grown and harvested at the time point of interest.

2. Dilute the starter culture 1:1000 in a 50 mL Erlenmeyer flask containing 25 mL of fresh liquid medium (the medium of interest).
3. Grow the culture in an orbital shaker at 200 rpm at 37°C. Monitor the growth by measuring the optical density at OD_{600} every hour. Refer to Figure 1 for information on the growth phase.

   ⚠ CRITICAL: Exponentially growing bacteria grown in LB are used for positive and negative controls, include six samples from this phase (OD_{600} = 0.4–0.6), additionally to the other samples of interest.

4. Once the growth phase of interest is reached, calculate the volume of bacteria to harvest using the formula (f1).

$$vol\ (ml) = \frac{2}{OD_{600}}$$

   ⚠ CRITICAL: It is crucial to work with a consistent number of bacteria to ensure that experimental conditions are consistent and reproducible among experiments. To achieve this, normalize the bacteria numbers by measuring the OD_{600}, as indicated in the formula above.

Cell fixation – Day 1

Timing: 1 h

In this section, samples are fixed.

Fixation can be performed using either a standard method or a direct method.

5. Collect the volume of bacteria culture as calculated above and fix the cells in 3.7% formaldehyde using either the standard method or the direct method.
   a. For the standard method, pellet the bacteria at 5,000×*g* for 5 min at 20°C–25°C, then resuspend in 200 μL of 3.7% formaldehyde.
   b. For the direct method, add 16% formaldehyde directly to the liquid culture to achieve a final concentration of 3.7%.

   Note: We recommend the latter method when the number of samples to be collected is larger than 5, to avoid phase desynchronization of the samples.

6. Incubate for 30 min at 20°C–25°C with gentle shaking.

   Optional: The bacteria can now be plated on agar plates to confirm that the fixation process killed all the bacteria.

Cell permeabilization – Day 1

Timing: from 1 h up to 24 h

In this section, samples are permeabilized.

Cell permeabilization allows to create pores in the membrane and cell wall of the bacteria, for the rRNA-FISH probes to enter the cytosol.

7. Pellet the fixed bacteria for 5 min at 1,000×*g* at 20°C–25°C.
8. Remove the fixative and dispose of it in accordance with proper safety regulations.
9. Wash with 500 μL of 1xPBS.
10. Resuspend the pellet in 150 μL of Nuclease-Free H_2O.
11. Add 350 μL of 100% ethanol.

⚠ CRITICAL: Dissolving in water first prevents the formation of cell clumps.

12. Incubate for 1 h at 20°C–25°C with gentle shaking, or for 16–20 h at 4°C.

   Note: While this combination of fixation and permeabilization is effective for several Gram-negative and some Gram-positive bacteria,[3] optimization might be necessary with some bacteria. Modifications to this standard protocol include enzymatic digestion of the peptidoglycan wall and cell wall proteins (for Gram-positive *Listeria monocytogenes*),[4,5] the use of detergents like Triton X-100 to induce a channel-forming effect (for Gram-positive *Bacillus pumilus* spores),[6] and short-term incubation in lactic acid or hydrochloric acid to induce lipopolysaccharide release from the outer membrane (for Gram-negative *Pseudomonas aeruginosa*).[7]

   Pause point: The suspension can be stored at 4°C for up to a week before proceeding to hybridization.

Probe hybridization – Day 2

Timing: 2 h (short protocol); 12–16 h (long protocol)

In this section, samples are hybridized with the rRNA-FISH probes.

13. From each sample, take a 50 μL aliquot and pellet the permeabilized cells by centrifugation for 5 min at 1,000×*g* at 20°C–25°C. Store the remaining permeabilized cells at 4°C as backup.
14. Remove the supernatant.

Note: Depending on the type of bacteria being analyzed, the appearance of the pellet will vary: a pellet made of *E. coli* cells will spread along the vial, while a pellet of *S.*Tm cells will form a compact mass.

15. Hydrate the cells by resuspending in them in 2x SSC.
16. Incubate the suspension for 10 min at 20°C–25°C.
17. Prepare the staining buffers as follows, calculating 60 μL of staining solution per sample.
    a. General staining buffer:
        i. Pipette 1.2 μL of the 16S 100 μM stock solution in a new vial.
        ii. Add 53.8 μL of the hybridization buffer and mix well by gentle pipetting to avoid bubble formation.
    b. 16S-pre16S staining buffer:
        i. Pipette 1.2 μL of the 16S 100 μM stock solution in a new vial.
        ii. Add 1.2 μL of the pre-16S 100 μM stock solution.
        iii. Add 52.6 μL of hybridization buffer and mix well by gentle pipetting to avoid bubble formation.
    c. 16S-23S staining buffer:
        i. Pipette 1.2 μL of the 16S 100 μM stock solution in a new vial.
        ii. Add 1.2 μL of the 23S 100 μM stock solution.
        iii. Add 52.6 μL of hybridization buffer and mix well by gentle pipetting to avoid bubble formation.
    d. Negative control staining buffer:
        i. Pipette 1.2 μL of the Atto647 negative control 100 μM stock solution in a new vial.
        ii. Pipette 1.2 μL of the Cy3 negative control 100 μM stock solution in a new vial.
        iii. Add 52.6 μL of the hybridization buffer and mix well by gentle pipetting to avoid bubble formation.
    e. 16S rRNA compensation control staining buffer:
        i. Pipette 1.2 μL of the 16S 100 μM stock solution in a new vial.
        ii. Add 53.8 μL of the hybridization buffer and mix well by gentle pipetting to avoid bubble formation.
    f. 23S rRNA compensation control staining buffer:
        i. Pipette 1.2 μL of the 23S 100 μM stock solution in a new vial.
        ii. Add 53.8 μL of the hybridization buffer and mix well by gentle pipetting to avoid bubble formation.
18. Slowly resuspend the cells in 50 μL of the staining buffer, taking care to avoid the formation of bubbles. Include the following samples:
    a. Positive control for localization: stain an exponential phase sample with 16S-pre16S staining buffer.
    b. Positive control for correlation: stain an exponential phase sample with 16S-23S staining buffer.
    c. Negative control: stain an exponential phase sample with the negative control staining buffer.
    d. 16S rRNA compensation control: stain an exponential phase sample with 16S rRNA compensation staining buffer.
    e. 23S rRNA compensation control: stain an exponential phase sample with 23S rRNA compensation staining buffer.
    f. DAPI compensation control: resuspend an exponential phase sample with hybridization buffer only without probes.

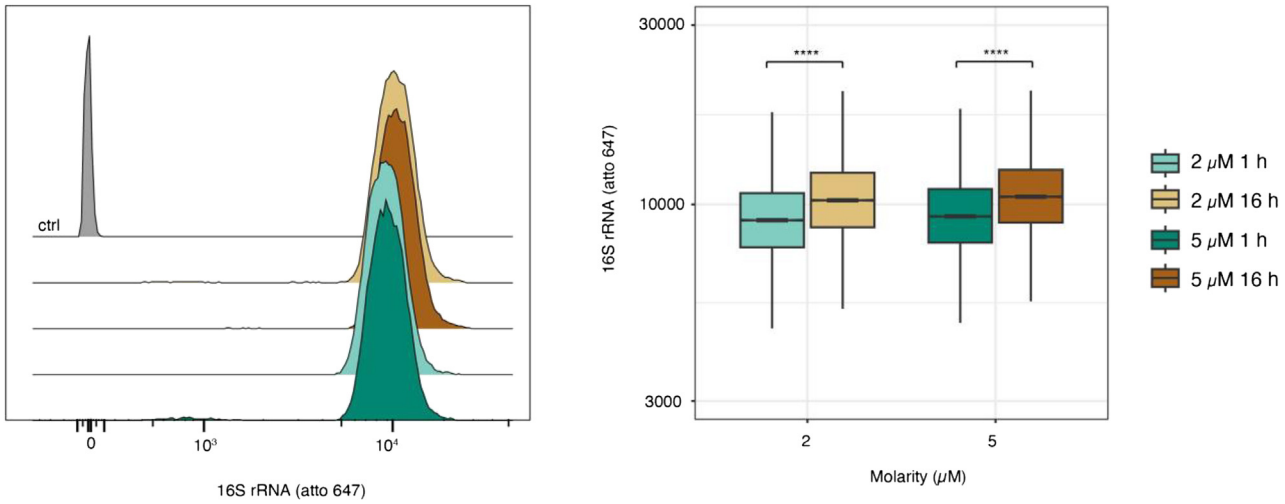

Figure 2. Density histograms (on the left) and boxplots (on the right) show 16S rRNA-FISH total fluorescence intensity of individual *S.*Tm cells measured by flow cytometry

Samples were incubated with 2 or 5 μM of the 16S probe, for 1 h (in light and dark green, respectively) or 16 h (in light and dark brown, respectively) (n = 50,000). Control sample is shown in the density plot as reference (in gray). Unpaired t-test: ****, $p < 0.0001$.

g. Remaining samples: stain with the general staining buffer.

19. Incubate for 1 h (short protocol) or 12–16 (long protocol) h at 37°C.

Note: Previous studies in mammalian systems have investigated the efficacy of shorter incubation times, demonstrating that a 5-minute hybridization period was sufficient when using a 20-fold increase in probe concentration and replacing PFA and ethanol with methanol during the fixation and permeabilization step.[8] While this accelerated protocol could potentially be applied to bacterial cells, it is not recommended due to the significant cell shrinkage methanol induces.[9] We have tested shorter incubation times (1 hour versus 16 hours) with two different probe concentrations (2 and 5 μM). We observed that 1 hour incubation is also sufficient to stain all cells, albeit at slightly lower levels, while increasing the probe amounts from 2 to 5 μM did not result in higher staining levels (Figure 2).

Pause point: The hybridized cells can be stored for up to a month at 4°C protected from light before proceeding to data acquisition.

Washing – Day 3

Timing: 3 h

In this section, samples are washed to remove unbound probes.

20. Transfer 10 μL of the hybridized samples in a new microcentrifuge vial or plate. For handling eight or more samples, using a PCR 96-well plate is recommended to facilitate the washing steps.
21. Perform the first wash with 200 μL of washing buffer and mix thoroughly by pipetting.
22. Pellet 5 min at 1,000×*g* at 20°C–25°C, and discard the supernatant.
23. Wash with 200 μL of washing buffer, mixing thoroughly by pipetting, and then incubate at 37°C for 30 min. Pellet 5 min at 1,000×*g* at 20°C–25°C, and discard the supernatant.
24. Repeat the step above.

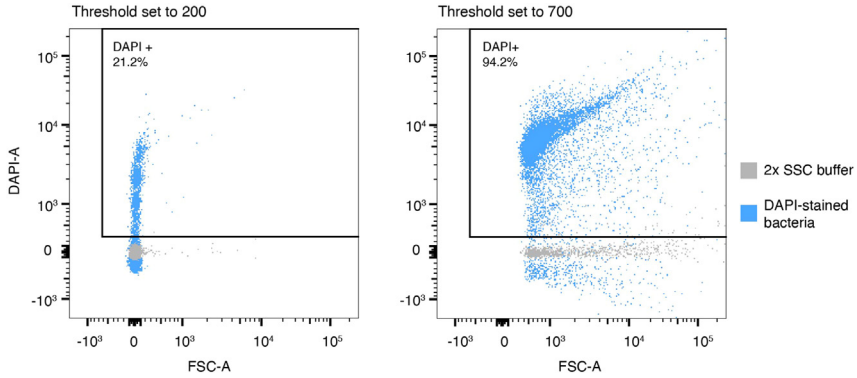

Figure 3. Threshold optimization allows the removal of unwanted events (debris and particles)

Threshold setting was optimized using DAPI-stained bacteria (in blue) and FACS buffer (in gray) to distinguish true bacterial events from debris and particles. When the threshold was set at 200, only 21.2% of the recorded events were DAPI-positive, indicating that most of the events were not cells (left panel). When the threshold was increased to 700 (right panel), the specificity of detection greatly improved, with 94.2% of the events representing bacteria. A higher threshold (1000; data not shown) resulted in loss of events.

25. Wash all the samples excluding the 16S and 23S compensation controls with 200 µL of washing buffer containing DAPI, mixing thoroughly by pipetting, and incubate at 37°C for 30 min. Pellet 5 min at 1,000×*g* at 20°C–25°C, and discard the supernatant.
26. Wash the 16S and 23S compensation controls with 200 µL of washing buffer, mixing thoroughly by pipetting, and then incubate at 37°C for 30 min. Pellet 5 min at 1,000×*g* at 20°C–25°C, and discard the supernatant.
27. Wash all the samples with 2x SSC, mix thoroughly by pipetting, then pellet for 5 min at 1,000×g at 20°C–25°C, and discard the supernatant.
28. Prepare the mounting buffer (GLOX anti-fade buffer) by adding 1 µL of glucose oxidase and 1 µL of catalase to a 100 µL aliquot of mounting buffer without enzymes.
29. Resuspend the pellet in 50 µL of mounting buffer.

Data acquisition by flow cytometry– day 3

Timing: 30 min to 2 h (depending on the number of samples)

In this section, samples are acquired using flow cytometry.

30. Prepare 5 mL round bottom polystyrene tubes with 0.5 mL of 2x SSC.
31. Transfer 5 µL of the sample in mounting buffer into the polystyrene tubes.
32. Prepare a mixed sample composed of the positive control for correlation and the negative control samples.
33. Adjust the threshold value of the Forward Scatter (FSC) to 700 on a FACSAria III.

Note: The ideal threshold placement is between the cells of interest and the noise floor. Optimization of this parameter may be necessary, depending on the flow cytometer available. Comparative analysis of a DAPI-stained bacterial sample and a buffer-only control can facilitate the identification of the appropriate threshold value (see Figure 3).

34. Using a 70 µM nozzle and a Neutral Density Filter of 1 (ND1), analyze the mixed sample to select the appropriate voltage settings for Atto647 16S rRNA-FISH, Cy3 23S rRNA-FISH and DAPI, by selecting a voltage that place the negative population around 10^2 and the positive population below 10^5.
35. Begin gating following the order:

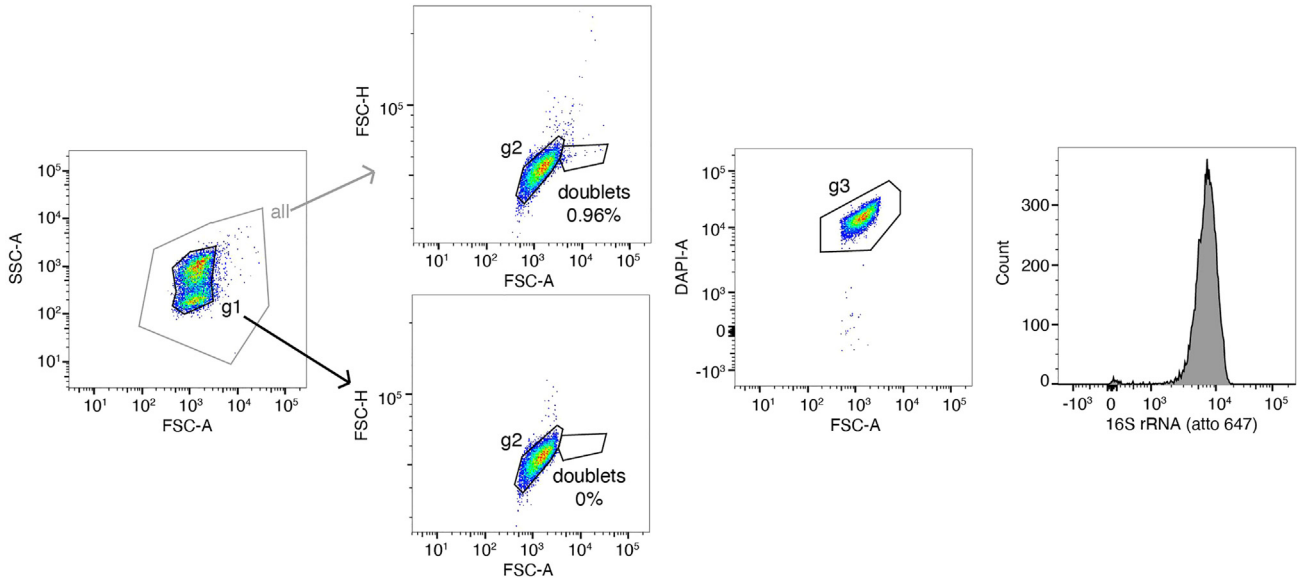

Figure 4. Gating strategy for selecting bacteria in flow cytometry

Bacteria are gated on FSC and side scatter SSC areas (g1), singlets are gated on FSC-A and FSC-H (g2), debris and particles are further removed using FSC and DAPI areas (g3). 16S rRNA signal is analyzed as histogram.

a. Use the FSC-Area (FSC-A) versus Side SCatter Area (SSC-A) parameters to select the bacterial population (g1) (Figure 4).
b. Use the FSC-A versus the FSC-Height (FSC-H) to select single cells and discard doublets (g2) (Figure 4).

Note: Visualizing flow cytometry data as density plots as opposed to dot plots helps discriminating doublet events already in the first gate (g1), by drawing the gate close to the high-density population. By doing so, most of the doublets are removed. When g1 is drawn including all events (depicted in gray as "all" in Figure 4), the population still contains doublet events, that can be further discriminated using the second gate (g2), as shown in Figure 4.

c. Select FSC-A versus DAPI to identify bacterial cells and discard remaining debris particles (g3) (Figure 4).
d. Select 16S as a histogram.

36. Analyze all the samples.
37. Run the compensation controls.

Experimental setup for flow cytometry acquisition

Laser	Filter	Range (nm)	Fluorophore used
Violet 405	450/40	430–470	DAPI
Yellow 561	582/15	575–590	Cy3
Red 633	660/20	650–670	Atto647

Data acquisition by microscopy – Day 3

Timing: 1–2 h (depending on the number of samples)

In this section, samples are acquired using microscopy.

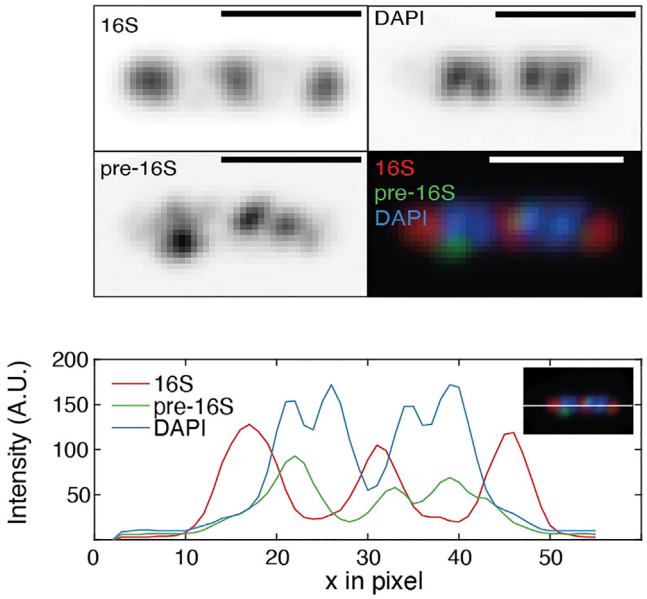

Figure 5. 16S rRNA signal localizes to cytosolic regions corresponding to rRNA loaded into ribosomes, as opposed to pre-16S rRNA colocalizing with DAPI and corresponding to newly transcribed rRNA

In the upper panel, representative microscopy image of an exponential phase *S.*Tm bacterium stained with 16S rRNA-FISH in red, pre-16S rRNA-FISH in green, and DAPI in blue. Single channels in black. Scale bar, 2.5 μm. In the lower panel, intensity profile of each fluorescent channel, color-coded as in the panel above. The intensity profile was measured across the horizontal axis of the cell as indicated by the white line. This data is presented in Figure S1 in Ciolli Mattioli et al., 2023.[1]

We used an inverted epifluorescence microscope (Eclipse Ti2-E, Nikon) equipped with a x100 NA 1.45 oil-immersion objective, filter sets (DAPI, GFP, Cy3, Cy5/Cy5.5), and a CMOS camera (Prime 95C, Photometrics), as previously described.[1] Data can be collected using any fluorescent microscope with RGB channels and filters to visualize red, green, and blue signals. Alternatively, a confocal microscope also can be used to observe signal localization.

38. Transfer 2 μL of the sample in mounting buffer on a Polysine coated microscope glass slide.
39. Cover the sample with a rectangular coverslip of thickness no. 1.
40. Keep the slides in the dark at 20°C–25°C for 1 h before imaging.
41. Set up a glass slide on a 100x oil-immersion objective lens.
42. Determine the optimal imaging settings, using exposure times that produce foci pixel values around 30% of the maximum pixel value of the camera (65,535 max value for a 16-bit camera). Avoid exposure times above 300 ms to avoid photobleaching.
43. Prepare image files for MATLAB analysis by selecting single bacterial cells aligned on the horizontal plane.
44. Use the following MATLAB script to analyze signal localization across the x-axis of the cell.

```
>I = imread('image.png');

>x = [0 size(I,2)];

>y = [size(I,1)/2 size(I,1)/2];

>c = improfile(I,x,y);
```

45. Use the following MATLAB script to visualize the results of the analysis.

```
>figure;
>subplot(2,1,1);
>imshow(I);
>hold on;
>plot(x,y,'r');
>subplot(2,1,2);
>plot(c(:,1,1),'r');
>hold on;
>plot(c(:,1,2),'g');
>plot(c(:,1,3),'b');
>xlabel('x in pixel');
>ylabel('intensity');
```

EXPECTED OUTCOMES

In bacteria with a low nucleoid-to-cytoplasm ratio, the localization of ribosomes is non-random, with enrichment observed in the nucleoid-free region of the cell.[10] To confirm that indeed ribosomes localize to the cytoplasm region,[10] we suggest using two different probes. One targeting mature 16S rRNA, which identifies mature ribosomes, and another targeting premature 16S rRNA, indicative of newly transcribed rRNA not yet assembled into ribosomes, and expected to localize to the nucleoid. As depicted in Figure 5, our imaging analysis shows a spatial segregation of mature 16S rRNA from the nucleoid, while the premature 16S rRNA exhibited spatial overlap with the DAPI-stained nucleoid.

In exponential phase, the growth rate of bacterial cells is linearly correlated with the abundance of ribosomes, which can be quantified by measuring the ratio of RNA to protein (RNA/protein ratio).[11] We suggest culturing the bacteria in different media compositions (e.g., M63 with glucose; M63 with casamino acids and glycerol; rich defined medium[12] with glycerol), where various growth rates are induced, resulting in distinct RNA/protein ratios for each growth condition.[13] Since rRNA-FISH also serves as a proxy for ribosome abundance, the measurements obtained through this method should show a linear correlation with RNA/protein ratio measurements, as shown in Figure 6.

rRNA is known to be stable only when incorporated into functional monosomes, which are composed of both the 30S and 50S subunits. We suggest using two distinct probes, to target specifically each ribosomal subunits: a probe targeting the 16S rRNA identifying the 30S subunit, and a probe targeting the 23S rRNA identifying the 50S subunit. The fluorescence intensities obtained from these two probes should exhibit a high degree of correlation when analyzed using rRNA-FISH, as shown in Figure 7.

In exponential phase, nutrients are not limiting and bacteria divide at the maximal rate characteristic for the given growth condition. Given the dependence of growth rate on protein synthesis and ribosome abundance,[11] exponentially growing bacteria are expected to show a uniform ribosome abundance per cell (as shown in Figure 8, in yellow). On the other hand, when *S.*Tm bacteria exit the exponential phase and enter a nutrient-limited state, a bimodal distribution of cells characterized by high and low levels of ribosomes is expected to emerge[1] (as shown in Figure 8, in gray).

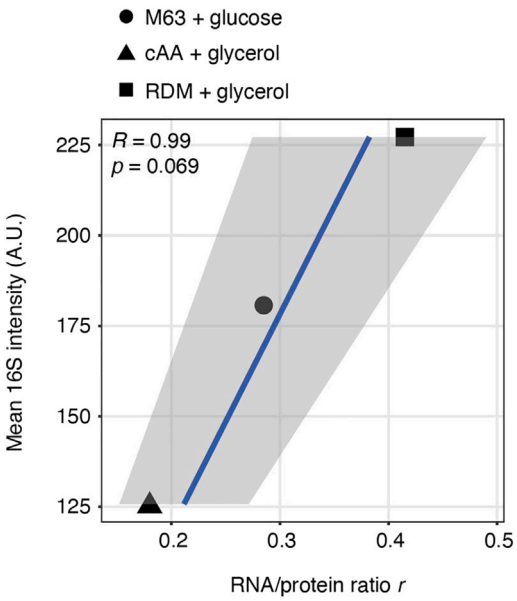

Figure 6. rRNA-FISH correlates with RNA/protein ratio measurements from literature

E. coli exponentially growing at different rates in different media is characterized by different RNA/protein ratios. Bulk averages of 16S rRNA measured by 16S rRNA-FISH and acquired by ImageStream are plotted against RNA/protein ratios, showing a linear correlation. Solid triangle, M63 with glycerol (0.5%, w/v) (growth rate $\lambda = 0.4\ h^{-1}$); solid circle, M63 + cAA (0.2% casamino acids, w/v) with glucose (0.5%, w/v)(growth rate $\lambda = 1\ h^{-1}$); solid square, RDM with glycerol (0.5%, w/v) (growth rate $\lambda = 1.31\ h^{-1}$). This data is presented in Figure S1 in Ciolli Mattioli et al., 2023.[1]

LIMITATIONS

In this protocol, we have described how to quantify ribosomes using a rRNA-FISH technique. The protocol has been optimized for *S.*Tm, and also tested in *S.* typhi, *E. coli*, and *S. aureus*. The 16S probe was designed to target a conserved region (nucleotides 337–354, located between hypervariable regions V2 and V3) of the 16S rRNA, and it is expected to anneal to the 16S of most bacteria, with the exception of those belonging to the Phylum Planctomycetota, as shown in Figure 9. The coverage of the probe across the different taxa was evaluated using TestProbe functionality of SILVA[14] (https://www.arb-silva.de/search/testprobe/) (see Table S1). Due to the broad annealing spectrum of the 16S probe, it cannot be used to discriminate specific bacterial species within a mixed sample. In contrast, the 23S probe was designed on a region that does not show the same level of conservation as the 16S locus, therefore its use is limited to certain bacterial phyla (see Figure 9; Table S1). The negative control probe was designed to not match on the *Salmonella* genome. We suggest the use of GuideFinder[15] as a tool to identify scramble sequences in different bacterial species.

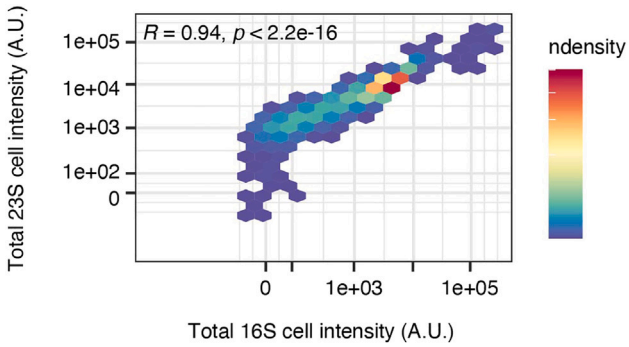

Figure 7. 16S signal and 23S signal highly correlate

2D distributions of *S.*Tm 16S and 23S fluorescence intensity areas measured by flow cytometry from exponentially growing bacteria, using DAPI+ gated population ($n = 20{,}000$ cells). This data is presented in Figure S1 in Ciolli Mattioli et al., 2023.[1]

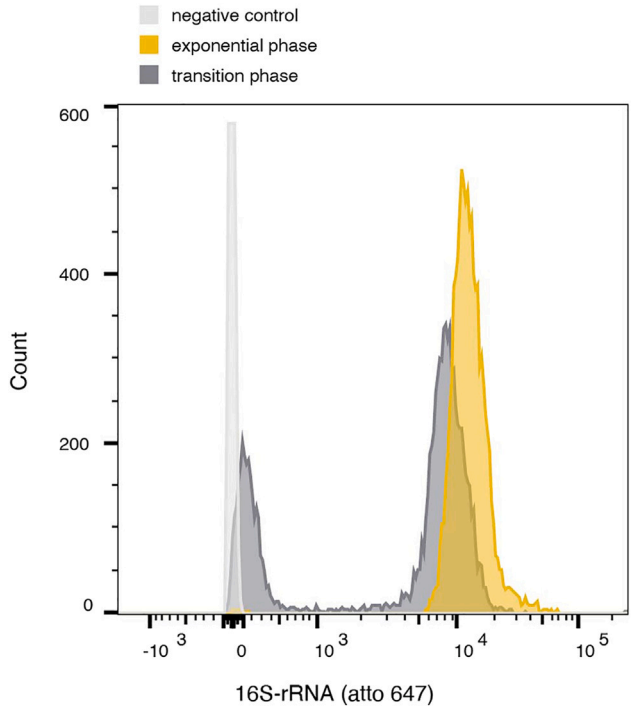

Figure 8. In exponential phase *S.*Tm cells have uniform levels of ribosomes, while during transition to stationary phase, ribosomal levels display a bimodal distribution

Density histograms show 16S rRNA-FISH total fluorescence intensity of individual S.Tm cells measured by flow cytometry during exponential growth (in yellow) and the transition from exponential growth to stationary phase (in dark gray) (n = 9,000). Non-stained bacteria (unstained control) are shown in light gray.

TROUBLESHOOTING

Problem 1

Lack of signal in the exponential phase control.

Potential solutions

- Contamination by RNases can lead to the loss of signal, as these enzymes degrade RNA and can thus compromise the integrity of the rRNA targeted by the FISH probes. Any reagent or material used needs to be carefully handled to avoid RNase contamination. Labware and surfaces should be clean with RNase away decontaminant before starting to work. Use Nuclease-Free disposable plasticware and filter tips. Gloves should be worn at all times.
- Failed permeabilization can also lead to lack of signal due to the exclusion of the probe from the cells. Using a membrane impermeable DNA-binding stain such as propidium iodide can help assess the success of the permeabilization step. Include a positive control (heat-killed bacteria, at 95°C for 5 min) and a negative control (alive bacteria).

Problem 2

Related to day 1: cell preparation.

Staining is very heterogeneous among different cells in exponential phase (see Figure 10 for an example).

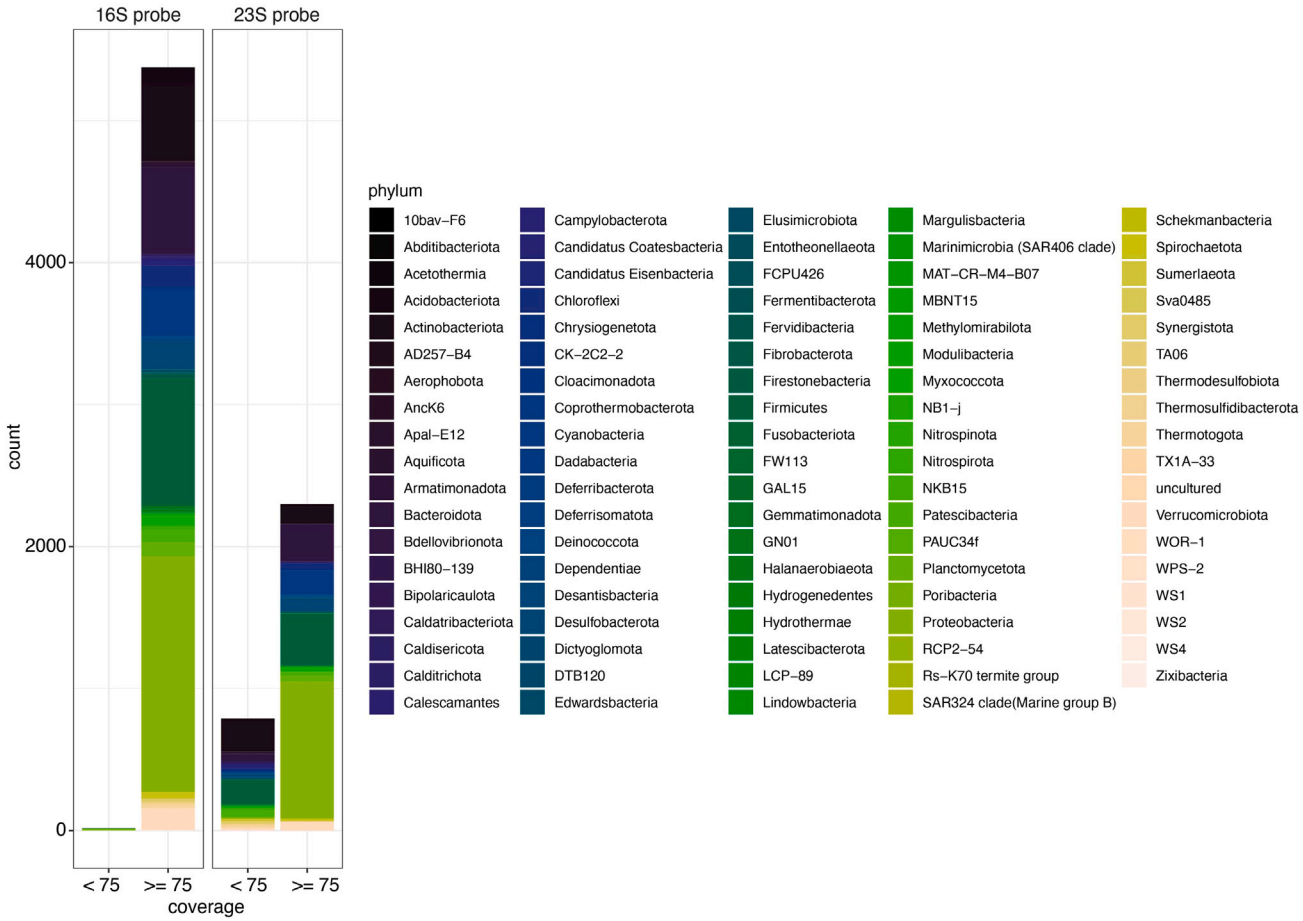

Figure 9. 16S probe shows high coverage on almost all bacterial Phyla

Phyla counts are shown, separately for low (< 75) and high (>= 75) coverage for 16S and 23S probes, separately. See also Table S1.

Potential solution

- Heterogeneous staining in exponential phase can happen when the ratio of cells to probe is biased toward the cells. To obtain a uniform staining the probe always needs to be in excess. To ensure an excess of probe, be sure to measure the volume of bacteria to take according to the formula f1, and to be in the readable photometric range according to the cell density meter used (0.3–1.99 for Ultrospec 10).
- Heterogeneous staining might also be caused by a high number of dead cells in the sample. To assess cell death in the samples before fixation, we recommend to assess cell viability by single cell colony forming unit.[1]

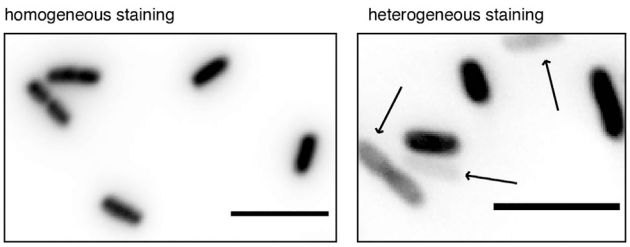

Figure 10. Examples of images with homogeneous rRNA staining on the left, and heterogeneous staining on the right

Cells with lower amount of probe are indicated with arrows. Scale bar, 5 μm.

Problem 3

Related to day 2: probe hybridization.

Staining is variable among replicates.

- Technical errors can cause fluctuations in fluorescence intensities as a result of differences in hybridization efficiency. This problem can be minimized by using an internal spike-in control population, which is added before hybridization and used to normalize the 16S intensities within each sample. The spike-in control can be a GFP-positive population, where the GFP signal is used to discriminate the spike-in population from the sample.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Roi Avraham (roi.avraham@weizmann.ac.il).

Technical contact

Further information regarding protocol details should be directed to the technical contact, Camilla Ciolli Mattioli (Camilla.ciolli-mattioli@weizmann.ac.il).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate or analyze any dataset or code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2024.103137.

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AUTHOR CONTRIBUTIONS

R.A. and C.C.M. conceptualized the methodology. C.C.M. developed the protocol and wrote the original draft. C.C.M. conducted the experiments. C.C.M. analyzed the data. All authors reviewed and revised the manuscript. C.C.M. and R.A. acquired the funding support and supervised the study.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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