

Imaging rRNA methylation in bacteria by MR-FISH

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Abstract

Methylation of RNA is normally monitored in purified cell lysates using next-generation sequencing, gel electrophoresis or mass spectrometry as readouts. These bulk methods require the RNA from $\sim 10^4$ - 10^7 cells to be pooled to generate sufficient material for analysis. Here we describe a method – methylation-sensitive RNA *in situ* hybridization (MR-FISH) - that assays rRNA methylation in bacteria on a cell-by-cell basis, using methylation-sensitive hybridization probes and fluorescence microscopy. We outline step-by-step protocols for designing probes, *in situ* hybridization and analysis of data using freely-available code.

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1 Introduction

Detection of methylated RNA bases has been practiced for 60 years [1] and methylation of ribosomal RNA (rRNA) has been known about since 1964 [2]. There are therefore very longstanding methods that are able to locate methylated bases (Fig. 1). These involve digesting cellular RNAs to individual nucleosides which are separated by chromatography [originally thin layer chromatography (TLC), now more commonly high-performance liquid chromatography (HPLC) or liquid chromatography–mass spectrometry, (LCMS)] to identify modified bases. The digestion process deletes sequence context, which can be recovered by reverse transcription assays coupled with gel electrophoresis [3]. The recent renewed interest in RNA methylation in epitranscriptomics has been triggered by a range of new next-generation sequencing methods that use immunoprecipitation to locate specific modifications in the transcriptome [4]. However, these state-of-the-art techniques as well as historic bulk methods have detection limits in the femtomole to picomole range, which even for abundant species like rRNAs therefore report the average methylation state of $\sim 10^4$ - 10^7 cells [5, 6]. As more methylations are discovered by these techniques, interest grows in new site-specific methods to detect modified bases in order to study their roles in biology [7]. Recently, we described a method called “methylation-sensitive RNA fluorescence *in situ* hybridization” (MR-FISH), which detects rRNA methylations in single cells (Fig. 1) [8]. We have shown that MR-FISH can detect three different methylations of rRNA [*N*⁶-dimethyladenosine (m^6_2A), 1-methylguanosine (m^1G) and 3-methyluridine (m^3U)], quantify antibiotic-resistant bacteria in mixtures of cells, and simultaneously detect multiple methylations using multicolor fluorescence imaging.

MR-FISH assays post-transcriptional modifications inside fixed cells rather than in extracted RNA, which greatly enhances sensitivity: the concentration of rRNA inside exponentially-growing bacteria can reach $>50 \mu M$ [9], so methylation-sensitive fluorogenic probes generate a signal within each cell that can be detected with a basic fluorescence microscope. Methylation can be detected by hybridization because methyl groups on the Watson-Crick faces can impair base pairing [10, 11]. To maximize this thermodynamic discrimination, MR-FISH uses Molecular Beacons [12] as hybridization probes. Molecular Beacons are more sensitive to single-base changes than linear probes, because they can retain

their hairpin structure in preference to making a less-stable duplex with a mismatched nucleic acid [13]. We use two such probes in MR-FISH, labeled with different fluorophores: one methylation-sensitive probe and a second probe that binds rRNA at a sequence remote from the modification site, that does not undergo methylation. This methylation-insensitive probe acts as an internal calibrant, meaning that the two-color ratio of each cell - rather than the intensity of the methylation-sensitive probe alone - indicates the extent of methylation. This ratio distinguishes between cells with a high proportion of methylated rRNA and cells which simply have a low concentration of rRNA. Fluorescence images are analyzed using custom software, which has four main functions: i) ensuring registration of the images in each color channel by cross-correlation; ii) generating a binary mask by thresholding the image generated by hybridization of the methylation-insensitive probe; iii) selecting single, isolated bacteria by tracing continuous regions of 1's in the binary image to exclude aggregates of bacteria; iv) integrating the fluorescence intensity of selected single-cells in each color channel and calculating the two-color ratio. The entire analysis procedure is fully automated and does not require the user to select control areas or individual bacteria, and can return information about the methylation status of thousands of single cells in minutes.

2 Materials

2.1 DNA probes

1. Select suitable fluorophore/quencher pairs for your fluorescence microscope. Table 1 lists fluorophore/quencher pairs we have used for MR-FISH, but more comprehensive lists for Molecular Beacons have been compiled [14]. Order Molecular Beacons (synthesized on the 0.2 μmol scale and purified by double HPLC) and any helper probes oligonucleotides (synthesized on either 0.2 or 1 μmol scale and purified by HPLC) from your preferred oligonucleotide supplier (see section 3.1 for guidance on probe design).
2. DEPC-treated water. Add 1 mL DEPC (0.1% by volume) to 1 L of 18.2 M Ω /cm water and stir vigorously for 2 h. Remove stirrer bar, filter through 0.2- μm syringe filter into 50 mL centrifuge tubes and autoclave for 2 h.
3. Dissolve lyophilized Molecular Beacons and unlabeled helper probes in DEPC-treated water to concentrations of 20–100 μM and confirm by OD₂₆₀. Divide into 20- μL aliquots and store at $-20\text{ }^{\circ}\text{C}$ until required. Defrosted aliquots can be stored for $4\text{ }^{\circ}\text{C}$ for up to two weeks; we avoid freeze-thaw cycles.

2.2 Bacterial cultures

1. LB broth: Weigh 25 g of LB broth powder and transfer to a 1 L beaker. Add ~ 800 mL of deionized water and dissolve by magnetic stirring. Remove the stirrer bar and add deionized water to a total volume of 1 L. Using a 100 mL measuring cylinder, aliquot into 10×100 mL in 100 mL Schott bottles and autoclave for 2 h.
2. LB agar plates: Weigh 2.5 g of LB broth powder and transfer to a 100 mL beaker. Add ~ 80 mL of deionized water and dissolve by magnetic stirring. Remove the stirrer bar and add deionized water to a total volume of 100 mL. Weigh 1.6 g of agar powder and transfer to a 100 mL Schott bottle. Add the 100 mL of LB broth, shake gently to disperse the agar and autoclave for 2 h. The agar

will not dissolve until the suspension is autoclaved. When the solution has cooled to 55 °C, pour 20 mL into a 9 cm diameter petri dish and allow to set by cooling to room temperature. Store plates at 4 °C for up to 1 week.

3. 50% (v/v) Glycerol solution: Weigh out 62.5g of glycerol into a 100 mL Schott bottle. Add 50 mL of deionized water, mix by gentle shaking and autoclave for 2h.
4. Bacterial Glycerol Stock: Transfer 0.5 mL of liquid bacterial culture to a 1.5 mL microcentrifuge tube. Add 0.25 mL of 50% glycerol and mix using a vortex shaker. Snap freeze the sample in liquid nitrogen and store at -80 °C.
5. Phosphate buffered saline (PBS): Weigh 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na₂HPO₄, 0.24 g of KH₂PO₄ and transfer to a 1 L volumetric flask. Add ~800 mL of deionized water and dissolve by magnetic stirring. Adjust the pH to 7.4 with 2 M HCl. Remove the stirrer bar and add deionized water to a total volume of 1 L. Transfer to a 1 L Schott bottle and autoclave for 2 h.
6. 1 M Sodium hydroxide: Gently stir 800 mL of deionized water using magnetic stirring. Carefully add 40 g of sodium hydroxide pellets making sure the solution does not get too hot. Once dissolved, allow the solution to cool and adjust the volume to 1 L.
7. 4% Formaldehyde solution in PBS: Add ~800 mL of PBS to a glass beaker and add a stirrer bar. In a fume cupboard heat to 60 °C on a hot plate and stir using magnetic stirring, taking care that the solution does not boil. Add 40 g of paraformaldehyde powder. The powder may not immediately dissolve. Slowly raise the pH by adding 1 M sodium hydroxide dropwise from a pipette until the solution clears. Allow the solution to cool, then check the pH using a pH meter and adjust the pH to 6.9 using 2 M HCl. Transfer the solution to a 1 L volumetric flask and adjust the volume to 1 L with PBS. Filter through a 0.2- μ m syringe filter into 50 mL centrifuge tubes (replacing the filter if the back pressure increases to a point where filtration becomes difficult).
Tip. Best results are achieved if the 4% formaldehyde solution is freshly prepared, but it can be stored at -20 °C for 1 month. Be sure to thaw thoroughly before use. **Caution:** Paraformaldehyde

is toxic and a suspected carcinogen. Always conduct a thorough risk assessment and handle in a fume cupboard wearing gloves, eye protection and a lab coat.

2.3 In situ hybridization

Prepare stock solutions of all buffer components (except formamide) for FISH using 18.2 MΩ/cm water, treat with diethylpyrocarbonate (DEPC) to inactivate RNases and then autoclave to decompose DEPC prior to use. Use certified RNase-free microcentrifuge tubes and pipette tips and clean bench surfaces with RNaseZAP™ according to the manufacturer's instructions.

1. DEPC-treated water.
2. 0.2 M Tris-HCl stock solution: Weigh 31.52g Tris-HCl and transfer to a 1 L volumetric flask. Add water to a volume of 900 mL. Dissolve by magnetic stirring and adjust pH to 7.5 using 6 M HCl. Add 10 mL DEPC (1% by volume, see Note 1) and stir vigorously for 2 h. Remove stirrer bar and make up to 1 L with water. Filter through a 0.2-µm syringe filter into 50 mL centrifuge tubes (replacing the filter if the back pressure increases to a point where filtration becomes difficult) and autoclave for 2 h.
3. 4.5 M NaCl stock solution: Weigh 263.0 g NaCl and transfer to a 1 L volumetric flask. Add water to a volume of ~900 mL. Dissolve by magnetic stirring, add 1 mL DEPC (0.1% by volume) and stir vigorously for 2 h. Remove stirrer bar and make up to 1 L with water. Filter through a 0.2-µm syringe filter into 50 mL centrifuge tubes (replacing the filter if the back pressure increases to a point where filtration becomes difficult) and autoclave for 2 h.
4. 1% Sodium dodecyl sulfate (SDS) stock solution: Weigh 0.5 g SDS and transfer to a 50 mL volumetric flask. Add water to a volume of 45 mL. Dissolve by magnetic stirring, add 50 µL DEPC (0.1% by volume) and stir vigorously for 2 h. Remove stirrer bar and make up to 50 mL with water. Filter through a 0.2-µm syringe filter into 15 mL centrifuge tubes and autoclave for 2 h.

5. Hybridization buffer: 0.9 M NaCl, 0.02 M Tris, pH 7.5, 0.01% SDS, 20% v/v formamide (see Note 2). Mix 3 mL of 4.5 M NaCl stock solution, 1.5 mL of 0.2 M Tris-HCl stock solution, 150 μ L of 1% SDS stock solution, 3 mL of formamide (\geq 99.5%, ACS Reagent Grade, Sigma Aldrich) and make up to 15 mL with DEPC-treated water. Filter through a 0.2- μ m syringe filter into a 15 mL centrifuge tube (replacing the filter if the back pressure increases to a point where filtration becomes difficult). **Caution:** formamide is a suspected carcinogen, may damage fertility or the unborn child and may cause damage to organs through prolonged or repeated exposure. Always conduct a thorough risk assessment and handle in a fume cupboard, wearing gloves, eye protection and a lab coat.
6. Wash buffer B: 0.9 M NaCl, 0.02 M Tris, pH 7.5, 0.01% SDS. Mix 3 mL of 4.5 M NaCl stock solution, 1.5 mL of 0.2 M Tris-HCl stock solution 150 μ L of 1% SDS stock solution and make up to 15 mL with DEPC-treated water. Filter through a 0.2- μ m syringe filter into a 15 mL centrifuge tube (replacing the filter if the back pressure increases to a point where filtration becomes difficult).
7. Imaging buffer: 0.025 M NaCl, 0.02 M Tris, pH 7.5. Mix 83 μ L of 4.5 M NaCl stock solution, 1.5 mL of 0.2 M Tris-HCl stock solution and make up to 15 mL with DEPC-treated water. Filter through a 0.2- μ m syringe filter into a 15 mL centrifuge tube (replacing the filter if the back pressure increases to a point where filtration becomes difficult).
8. Coverslips: 22 \times 22 mm, thickness 0.13-0.17 mm, pre-cleaned with argon plasma for at least 30 minutes.
9. Frame-Seal slide chamber: 9 \times 9 mm
10. Mounting medium: Hydromount slide mounting medium

2.4 Fluorescence Microscopy

1. To read out the MR-FISH signal in individual cells, we image bacteria using multicolor widefield epifluorescence microscopy (ideal laser lines are 488, 561 and 640 nm), equipped with a suitable objective (see Note 3).
2. The different emission colors (e.g. green and red fluorescence) can be recorded simultaneously if colors can be spectrally separated, or sequentially, depending on the microscope used. Fluorescence emission was recorded on an EMCCD camera in our previous work, however sCMOS cameras would be equally well suited.

2.5 Image Analysis

1. A computer with >8GB memory, running MATLAB R2010b (or later releases) and the following MATLAB tool boxes: Image Processing, Curve Fitting and Statistics.
2. Custom analysis software; the ready-to-run code is available for download on github: <https://github.com/kganzinger/Analysis-Software-for-in-situ-hybridization-data-in-single-cells/>.
The code is actively maintained, so any bugs should be reported via github or by emailing K.A.G.

3 Methods

3.1 Probe design

1. Select a 15-20 nt section of the rRNA target sequence with the modification site in the center and generate its reverse complement as a probe sequence. As with Molecular Beacons used to detect single mismatches, Molecular Beacons for MR-FISH should be designed with relatively short probe sequences to maximize the thermodynamic effect of methylation on hybridization. Simulate the properties of the probe-target duplex using the DINAMelt [15] Web Server (<http://unafold.rna.albany.edu/?q=DINAMelt/Hybrid2>), using the following parameters:
 - o temperature range: 0-100 °C (these are the default settings)
 - o NA (nucleic acid) type: DNA (this is the default setting, see Note 4)

- Salt Conditions: $[Na^+] = 1 \text{ M}$ and $[Mg^{++}] = 0 \text{ M}$ (these are the default settings)
- Initial concentrations: $[A_0]$ and $[B_0] = 1 \mu\text{M}$

Molecular Beacons which we have successfully used to discriminate between methylated and unmethylated RNA return ΔG values in the range of 22-35 kcal mol⁻¹ for the probe-target duplex. ΔG values for Molecular Beacons that we have used to discriminate single methylations fall within a narrower range (22-27.5 kcal mol⁻¹), but we have not systematically studied this.

2. Add 5-6 self-complementary nucleotides comprising either all Gs and Cs or containing one A:T pair to each end of the probe sequence, e.g. 5'-CCGCCX¹X²X³X⁴X⁵X⁶X⁷**Z**X⁸X⁹X¹⁰X¹¹X¹²X¹³X¹⁴GGCGG-3', where the underlined section forms the stem of the Molecular Beacon, **Z** is complementary to the modification site and Xs are complementary to the bases either side of the modification site. Avoid stem designs with a G, and especially runs of consecutive Gs, next to the fluorescent label, because these can quench fluorescence and reduce the signal in FISH; we normally attach the dye at the 5' end of the Molecular Beacon, but most dyes can be added at either the 5' or 3' end if required.
3. Use the mfold [16] webserver (<http://unafold.rna.albany.edu/?q=mfold>) to simulate the secondary structure of the Molecular Beacon you designed, using a folding temperature of 37 °C, $[Na^+] = 1 \text{ M}$ and $[Mg^{++}] = 0 \text{ M}$ (these are the default settings). In our experience, the ideal Molecular Beacon folds into a hairpin loop where the stem is formed by the intended 5-6 nucleotides, with a ΔG of 2.5-7 kcal mol⁻¹ (and 5.5-7 kcal mol⁻¹ for discrimination of single methylations) and minimal self-complementarity in the loop, but we have not systematically studied this.
4. Because rRNA is highly structured, it is common to find substantial secondary structure in the probe section of the Molecular Beacon. Isolated structures with 2-3 base pairs are not normally a problem, but self-complementarity which extends the stem length beyond 6 base pairs can inhibit hybridization and should be eliminated if possible. Various strategies can achieve this, but typically this will involve removing nucleotides from the original or extended stem. For example, if simulation reveals the structure to be 5'-

CCGCCX¹X²X³X⁴X⁵X⁶X⁷ZX⁸X⁹X¹⁰X¹¹X¹²X¹³X¹⁴GGCGG-3' due to self-complementarity between X¹/X¹⁴ and X²/X¹³, alternative designs might include 5'-CCGX¹X²X³X⁴X⁵X⁶X⁷ZX⁸X⁹X¹⁰X¹¹X¹²X¹³X¹⁴CGG-3',
CCGCX²X³X⁴X⁵X⁶X⁷ZX⁸X⁹X¹⁰X¹¹X¹²X¹³GCGG-3' or
CCGCCX³X⁴X⁵X⁶X⁷ZX⁸X⁹X¹⁰X¹¹X¹²X¹³X¹⁴GGCGG-3'. Repeat step 3 until the stem is formed by the intended 5-6 nucleotides, with a ΔG of 2.5-7 kcal mol⁻¹ (and 5.5-7 kcal mol⁻¹ for discrimination of single methylations). This can require multiple iterations (see Note 5).

5. Estimate the extent of discrimination between methylated and unmethylated targets with the DINAMelt Web Server (<http://unafold.rna.albany.edu/?q=DINAMelt/Hybrid2>) by simulating melting of your full Molecular Beacon sequence with the target sequence, using the following parameters:

- o temperature range: 0-100 °C (these are the default settings)
- o NA (nucleic acid) type: DNA (this is the default setting, see Note 4)
- o Salt Conditions: [Na⁺] = 1 M and [Mg⁺⁺] = 0 M (these are the default settings)
- o Initial concentrations: [A₀] and [B₀] = 1 μM

Repeat this after modifying the target or Molecular Beacon sequence to have a C:A or G:T mismatch at the methylation site. The smallest difference in simulated melting temperature between the fully matched and mismatched Beacon-target duplexes (labeled AB in the generated plot) for Molecular Beacons we have successfully used in MR-FISH is >15 °C. If the difference is much smaller than this in your simulation, we suggest modifying the Beacon design, by increasing the stability of the stem relative to the stability of the loop section, repeating steps 3-5. This can require several iterations.

6. Helper DNA oligonucleotides that hybridize adjacent to the rRNA targeted by the Molecular Beacon can be used to increase hybridization efficiency if required (e.g. in case of low signal in FISH) [17]. This is more likely when the methylation site is in a relatively inaccessible region of the ribosome [18, 19]. To design these, select the 15-20 nt of the rRNA target sequence

immediately adjacent to each end of the Molecular Beacon's binding site and generate their reverse complement to give a helper probe sequence. Estimate the stability of the duplex made by the helper probes with the DINAMelt Web Server (<http://unafold.rna.albany.edu/?q=DINAMelt/Hybrid2>) by simulating melting of each probe with their reverse complement, using the following parameters:

- temperature range: 0-100 °C (these are the default settings)
- NA (nucleic acid) type: DNA (this is the default setting, see Note 4)
- Salt Conditions: $[Na^+] = 1 \text{ M}$ and $[Mg^{++}] = 0 \text{ M}$ (these are the default settings)
- Initial concentrations: $[A_0]$ and $[B_0] = 1 \mu\text{M}$

Helper oligonucleotides that we have successfully used for MR-FISH return ΔG values of 30-40 kcal mol⁻¹ when simulated with these parameters. Lengthen or shorten the DNA sequence if ΔG falls far outside this range. To generate a second pair of helper sequences, select the 15-20 nt of the rRNA target sequence immediately adjacent to each end targeted by the first set of helper probes and simulate melting as above, applying the same selection criterion (ΔG of 30-40 kcal mol⁻¹).

3.2 Bacterial culture

Use sterile technique up to the point of fixing the bacteria. For best results use a sterile laminar flow hood or if this is not available work close to a flame and avoid leaving cultures, broth or agar exposed to the laboratory environment for longer than necessary (see Note 6).

1. Grow isolated colonies of each strain you plan to use to generate biological replicates. Using a single use sterile plastic loop, streak out a glycerol stock onto an LB agar plate and incubate at 37 °C overnight for 12 – 18 h.
2. Prepare a starter culture. Aliquot 10 mL of sterile LB broth into sterilized glass universal tubes. Prepare three tubes per bacterial strain. Using a single-use sterile plastic loop, pick a well isolated

single bacterial colony from the cultured agar plate and dip the loop into one of the 10 mL aliquots of the LB broth. Repeat so you have three starter cultures per strain. Incubate these at 37 °C overnight for 12 – 18 h on an orbital shaker at 180 rpm.

3. Inoculate experimental culture. Aliquot 50 mL of sterile LB broth into sterilized, Pyrex 250 mL conical flasks. Prepare one flask per starter culture. Using a clean, sterile pipette tip, add 0.5 mL of your starter culture. Incubate at 37 °C until the culture reaches mid-log phase (see Note 7).
4. Inactivate (i.e. fix) bacteria with formaldehyde. When the OD₆₀₀ is between 0.4 and 0.8 units, pour 7.5 mL of bacterial culture into a clean, sterile 15 mL centrifuge tube. Add 7.5 mL of 4% formaldehyde in PBS and leave the resulting suspension at room temperature for 90 min.
5. Wash inactivated bacteria. Centrifuge the suspension for 10 min at 4,000 g to pellet the inactivated cells. Carefully pour off the supernatant and invert the tube over a piece of clean tissue to remove the residual liquid. Add 10 mL of PBS and gently suspend the pellet by pipetting the contents of the tube up and down. Repeat this twice to wash off the majority of the formaldehyde.
6. Storage of inactivated bacteria. Add 0.5 mL of PBS to the washed pellet of inactivated cells and suspend by gently pipetting up and down. Transfer to a clean, sterile 1.5 mL microcentrifuge tube. Add 0.5 mL of ethanol. The expected yield of 1 mL at $\sim 4.5 \times 10^9$ fixed *E. coli* per mL is sufficient for ~25-50 MR-FISH experiments. Store at -20 °C until required.

3.3 In situ hybridization

1. Pellet $\sim 1.35 \times 10^8$ fixed *E. coli* by centrifugation, resuspend in hybridization buffer, pellet by centrifugation and then resuspend in 20 μ L of hybridization buffer, containing 1 μ M methylation-insensitive Molecular Beacon and 1 μ M methylation-sensitive Molecular Beacon (in our case these are typically Alexa Fluor-647 and Alexa Fluor-488 labeled respectively, but these can be substituted with any pair of dyes spectrally resolved by your microscope, see Section 2.1). Incubate at 37 °C for 21 h (see Note 8) with agitation at 200 rpm.

2. Pellet cells by centrifugation, resuspend in 30 μL wash buffer A, then agitate at 200 rpm for 15 minutes at 37 °C.
3. Pellet cells by centrifugation, resuspend in 30 μL wash buffer B, then agitate at 200 rpm for 15 minutes at 37 °C.
4. Pellet cells by centrifugation and resuspend in 10 μL imaging buffer.
5. Attach a Frame-Seal slide chamber to a coverslip, add 5 μL of the cell suspension and a drop of mounting medium to the coverslip. Seal with another plasma-cleaned coverslip and allow to set for at least 30 minutes at room temperature before imaging (see Note 9).

3.4 Fluorescence Microscopy

1. Collect fluorescent images for each color, taking care to minimize bleed-through between channels by selecting appropriate filters and acquiring the channels sequentially if required.
2. Adapt the exposure time to the dynamic range of your camera and sample. If you are imaging a range of samples, start, if possible, with the samples for which you expect your maximal signal. Pick an exposure time (and camera gain) for which the average intensity of your signal is about half the dynamic range of your camera. Use 16-bit imaging if possible, as this will increase the dynamic range, allowing you to resolve smaller intensity differences between bacteria.
3. Collect sufficient images to yield >450 cells per sample for analysis after thresholding. In our experience, for samples prepared as in Section 3.3, this requires imaging an area of $\sim 0.7 \text{ mm}^2$, or approximately 50 fields of view of area $82 \times 164 \mu\text{m}$.

3.5 Image analysis

3.5.1 Data prerequisites:

1. Data need to be in .tif format with separate, equally-sized images for the different colors. ImageJ [20] can be used to convert images to .tif format. Set the exact file extension (.tif, .tiff, .stk) in

Graphical User Interface 1 (GUI-1) (see 3.5.7 and Fig. 2a). To get started, all the data from our previously published work is available on figshare and can be used as test data for the code [21].

2. Pairs or triplets of files need to be identified by their file name, which must start with an Arabic number (index), followed by the letter 'B' or 'R' (or 'Y' for three-color data) to indicate the color channel, e.g. 1B, 1R or 23R, 23B, 23Y (see Note 10 for instructions to change this nomenclature if desired).
3. Batch processing of a series of datasets is implemented; datasets need to be organized in directories (one directory per dataset). Since run time linearly increases with dataset size, we recommend optimizing the analysis on a single dataset first to select the analysis parameters before running larger datasets.

3.5.2 Running the software:

1. Copy the main function `Single_cell_FISH_analysis.m` and the source code (folder: "source codes") into a local directory.
2. Open the main function in MATLAB and run the code by pressing F5/"Run".
3. You will be prompted to set some parameters in four subsequent GUIs (Fig. 2, Table 2).
4. Use GUI-1 to set parameters that should be customized for each dataset analyzed (Fig. 2a). These include parameters that are specific to a dataset, such as the dataset name and image width. Other parameters that need adaptation are those used to detect bacteria in the fluorescent images, as this requires setting a threshold for detection that needs to be empirically optimized. All parameters are summarized and explained in Table 2. See Note 11 for a description of AND/OR/REDONLY criterion for detection, Note 12 for a detailed explanation on parameter range finding for the image thresholding step and Note 13 for a guide to the parameters used for discriminating between signals from single and aggregated bacterial cells. In GUI-2 (Fig. 2b), the user is asked to choose between running the analysis on multiple datasets in a row ('batch mode', "Yes") or just a single data file ("No"). GUI-3 and GUI-4 asks for the path to the parent directory in which the

data directories are located (Fig. 2c, batch mode) or the path to the data directory (Fig. 2d, single dataset analysis).

5. After confirmation of the settings in GUI-4, the software will run without user input unless an interactive option is selected in GUI-1 (i.e. if 'Display detected bacs' is set to 1).
6. *Analysis output (single dataset)*: The following plots are created as output: histograms of bacteria intensities for each color, histograms for coincidence between the channels (only meaningful if the 'OR' criterion is selected; we used the REDONLY criterion in our previous work); plot of $\ln(\text{red/green})$ intensity, or $\ln(\text{green/yellow})$ in the case of three-color experiments; text files with lists of intensity values for re-plotting using other software if desired. *Analysis output (batch mode)*. Additionally, the intensities and intensity ratios extracted from all datasets are combined and saved as a single excel spreadsheet after analysis of the last dataset.

4 Notes

1. The higher concentration of DEPC is required because Tris deactivates it *via* chemical reaction. Alternatively, dissolve Tris-HCl in DEPC-treated water (see 2.1.2)
2. The formamide concentration can be optimized to enhance signal and/or discrimination of methylation. Because formamide destabilizes the Molecular Beacon stem, rRNA secondary structure and the probe/target duplex, it is difficult to predict the appropriate concentration for a given set of probes and methylations, so we determine this empirically if needed (i.e. when 20% formamide gives low signal and/or poor discrimination of methylation).
3. We found that using an inexpensive, low-magnification objective (Plan Fluor 20× air, numerical aperture (NA) 0.50, Nikon) gave similar results as objectives with a higher numerical aperture (CFI Plan Apochromat λ 40× air, NA 0.95 and 60× Plan Apo TIRF oil immersion, NA 1.45, both by Nikon), with the added advantage of higher throughput due to the larger field-of-view obtained at lower magnifications.
4. There is currently no option for DNA/RNA hybrids.
5. Remove bases from the loop section with caution, as hairpin loops with very small loops may be too stable to hybridize with the target sequence; this will be reflected in a large ΔG of the hairpin loop structure. In our experience, loop lengths below 12 nucleotides can require stems shorter than 6 base pairs to hybridize. Shift methylation site from centre if needed or shorten the stem.

6. The protocol given is for culturing *Escherichia coli* BW25113. If culturing different strains or species, refer to any available data as the optimal culturing conditions may require modifications to the procedure given here. If the strain you are using includes an antibiotic resistance gene or is transformed with a plasmid containing an antibiotic resistance gene, you can include appropriate antibiotics at the appropriate concentration in the LB agar and LB broth.
7. Under optimal conditions, mid-log phase is usually reached in 2 – 3 h. The most convenient method to monitor bacterial growth is to transfer 1 mL of culture to a 1 cm pathlength cuvette using a sterile pipette and measure the absorbance at 600 nm (OD_{600}) in a spectrophotometer. Mid-log can be defined as when the OD_{600} is between 0.4 and 0.8 units.
8. The duration of *in situ* hybridization can be varied. Methylation can be discriminated within 0.5 h of hybridization [8], but this will vary depending on the hybridization kinetics of the specific probes used.
9. Cells which have had insufficient time to rehydrate in imaging buffer have poor morphology when imaged.
10. The letter code for the color can be changed in function "get_tiffs" or "get_tiffs_threecolours" in lines 10, 11, 12.
11. We use only the signal from the red channel for bacteria detection, since the red-labeled methylaton-insensitive probe is used to label ribosomes irrespective of methylation state. Using only the red channel thus minimized bias due to the expected large variation in fluorescence brightness in the green and yellow channels (reporting the methylation state). However, in different experiments it may be beneficial to first detect bacteria in in all channels, and then select objects based on whether they are recorded in *any* channel ('OR' criterion) or *all* channels ['AND' criterion; in principle, this can reduce the number of false positive events (*e.g.* detecting background rather than bacteria)]. This is already implemented for two color data.

12. To detect bacteria, first the 2D Fourier transform of the image is calculated and a lowpass filter is applied that removes frequencies corresponding to structures smaller than 8 μm . After this, the filtered image is obtained by reverse Fourier transformation, then converted to a binary image (mask) using a threshold intensity, set to be the mean single pixel intensity of the filtered red image plus 'initial threshold' times this value. This threshold is set by parameter 'initial threshold' in GUI-1 (Fig. 2a and Table 2). Pixels with intensity values below the threshold are set to zero and considered to be background, while pixels with values above the threshold are set to one and potential candidates for bacteria signals. Good values for the 'initial threshold' need to be determined empirically by visual inspection of the resulting mask and comparison with the original data, and will depend on the signal/noise ratio of the data (Fig. 3b-d). To display this mask during an analysis run, the parameter 'Display detected bacs' should be set to 1 (Table 2). The analysis will be paused as long as the image is displayed; press 'space' to continue to the next image of a dataset.

13. After obtaining an image mask, objects (bacteria) are detected in the next step by tracing continuous regions of ones in the binary image (mask). We have previously only used the signal from the red channel for bacteria detection, and apply this mask to both color channels, but it is also possible to create a mask from each channel individually. Segmentation of individual cells is directly dependent on the bacteria density in the sample, so this may require some optimization in the sample preparation step (see also section 3.3). However, we encountered aggregates of bacteria under any conditions we tried. Since these aggregates may consist of bacteria belonging to different strains, it is necessary to include further selection criteria for robust discrimination of single cells and cell aggregates. We have explored selection criteria based on region size, continuity and ellipticity. We expected that single cells should have, on average, a higher ellipticity than aggregates and cell debris, but we found that this criterion did not improve discrimination independently of the threshold ellipticity value used. However, this could

potentially aid discrimination for other bacteria, and this criterion can be activated if the parameter “threshold based on perimeter/area ratio” is set to 1 (Table 2). For our data analysis, we selected regions based on their size and continuity: regions were assumed to correspond to single bacteria if the area of the region is within a certain size range by setting a lower and upper threshold (parameter: ‘lower/upper threshold bac size’, Table 2). It is best to estimate the expected size distribution by manual analysis of the raw data, *e.g.* using ImageJ [20]. A size threshold of 1-10 μm^2 worked well for *E.coli* bacteria (Fig. 3e and 3f). The continuity criterion is set by the parameter ‘maximum num of empty px in ROI’ and it corresponds to the number of pixels with value zero contained within the region of interest (group of pixels of value 1, Table 2). Since these “empty” regions most likely correspond to the space between bacteria incorrectly merged into one single region, introducing this parameter helps to discriminate between groups of bacteria and individual cells. We required regions to contain fewer than 50 background pixels (value zero), corresponding to a total area of 5.15 μm^2 .

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Figure Legends

Fig. 1. Comparison of MR-FISH to existing methods for detecting base methylations.

Fig. 2. GUIs displayed on start-up of the MATLAB analysis software. **(a)** GUI-1 for entering user-defined parameters. Default options can be changed in the code. The red box highlights the parameters required for isolated object (bacteria) detection (explained in in notes 11-13). **(b)** GUI-2 to select mode for batch or single dataset analysis and the corresponding GUI-3 and GUI-4 to enter the path to the raw data to be analyzed **(c,d)**.

Fig. 3. Effect of thresholding parameters on image segmentation (detection of bacteria). **(a)** typical raw data; heat map goes from blue (low) to red (high) intensity. **(b-d)** Effect of parameter ‘initial threshold’ on converting the raw data into a binary image in which ROI (bacteria) are separated from the background. If the threshold is too low **(b,c)**, multiple bacteria in close proximity show up as a single ROI (red arrows). **(e-f)** Effect of parameters ‘lower/upper size threshold’ on selecting only ROIs corresponding to single bacteria. The range needs to be adjusted to the expected size distribution in order to achieve good discrimination (cf. also **(g)**). If the parameter ‘Display detected bacs’ is set to 1, these images (showing selected ROI by tracing them on the mask image generated) are displayed for each data file analyzed. **(g)** Distribution of bacteria sizes in **(a)** as determined from the analysis software after parameter optimization (see image in **e**).

Table 1. Fluorophore-quencher pairs for MR-FISH.

FLUOROPHORE	EXCITATION	EMISSION	QUENCHER
	WAVELENGTH (NM)	WAVELENGTH (NM)	
ALEXA FLUOR 488	495	519	DABCYL
FAM	490	514	DABCYL
CY3/CY3B	548/558	562/572	BHQ2
ALEXA FLUOR 647	650	668	BHQ1

Table 2. Explanation of user-dependent parameters for automated data analysis.

PARAMETER	EXPLANATION
'EXPERIMENT NAME'	defines name of experiment (identifier/name of results directory)
'CORRECTION OF CHANNEL OFFSET (NO=0,AUTO=1,FIXED=2)'	optional correction of positional offset between different color channels (offset in x and/or y) set to 0 for no correction set to 1 for automatic (cross-correlation-based) correction; calculated for each image pair/triplet set to 2 for correction using fixed values across all images. Opens another GUI prompting you to define the x/y offset in pixels.
'MASK CRITERION: AND (0) / OR (1) / REDONLY (2) '	set this parameter to 0 for detection of bacteria in all color channels and analysis according to the 'AND' criterion to 1 for detection of bacteria in all color channels and analysis according to the 'OR' criterion to 2 for detection of bacteria only in the red channel and analysis according to regions defined by the red channel for all colors (see also Note 11)
INITIAL THRESHOLD	empirical parameter that defines the binarization threshold for the images; binary images are required for object tracing/detection (see also Note 12)
SINGLE SIZE THRESHOLD? (0/1)	set to 1 to reject bacteria/objects solely based on a lower size limit, (see also Note 13)
THRESHOLD BASED ON PERIMETER/AREA RATIO?	set to 1 if additional discrimination based on the ratio of perimeter to area is desired (cf. methods of Ranasinghe et al. [8])

(0/1)	(see also Note 13)
'LOWER THRESHOLD BAC SIZE'	define lower limit for bacteria/object size (pixel ²) (see also Note 13)
'UPPER THRESHOLD BAC SIZE'	define upper limit for bacteria/object size (pixel ²) (see also Note 13)
'MAXIMUM NUM OF EMPTY PX IN ROI (REGION OF INTEREST)'	Maximum number of background pixels (value zero) that are allowed to be contained within an ROI (high values expected for grouping multiple bacteria into one ROI) (see also Note 13)
PATH TO RESULTS DIR	Define full path to results directory
DISPLAY DETECTED BACS (0/1)	set to 0 for non-interactive mode set to 1 for interactive mode: masks and detected bacteria are displayed for each image [user input (any key) is required to continue the analysis and move to the next image]
NUMBER OF COLOR CHANNELS (2/3)	set to 2 for two and to 3 for three color experiment
IMAGE WIDTH [PX]	Set the width of the image in pixels
'PIXEL SIZE [NM/PX]'	Set the pixel size of the imaging system in nanometers
DUAL VIEW (0/1)	The user is asked whether images were acquired with a dual-view in the emission path. If the user selects '1', the image is split, assuming that dual-view divides the [width] x [length] image into two [width]/2 x [length] images, one for each color. At present, this option is only implemented for two-color data.
IMAGE TYPE	Set the image format (.tif, .tiff or .stk)