Chapter 12

Quantitative Super-Resolution Imaging of Small RNAs in Bacterial Cells

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Abstract

We present a method for the quantification of small regulatory RNAs (sRNAs) in bacteria, by combining single-molecule fluorescence in situ hybridization (smFISH), super-resolved single-fluorophore microscopy, and clustering analysis. Compared to smFISH imaging with diffraction-limited fluorescence microscopy, our method provides better quantification for short and abundant RNA (such as sRNAs) in a small volume of bacterial cells. Our method can also be directly used for the quantification of messenger RNAs (mRNAs).

Key words Fluorescence in situ hybridization, Super-resolution microscopy, Clustering analysis, Small regulatory RNA

1 Introduction

Bacterial small regulatory RNAs (sRNAs) regulate gene expression and provide growth benefit for bacterial cells under stress conditions. They are typically 50–300 nucleotides in length, and are often associated with Hfq for their functions [1, 2]. In most common scenarios, sRNAs regulate gene expression posttranscriptionally by base pairing with target messenger RNAs (mRNAs) and thereby affect translation efficiency and/or mRNA stability [1, 2]. Single-molecule fluorescence in situ hybridization (smFISH) is widely used to count mRNAs at the single-cell level [3, 4], and has been applied to study gene expression, especially in eukaryotic systems. In smFISH, many, often dozens, fluorescently labeled DNA oligos are hybridized to different portions of the same mRNA to generate bright signal as diffraction-limited spots with conventional microscopy [4, 5]. However, direct quantification by counting spots from smFISH signal cannot be applied to bacterial sRNAs due to their short length that limits the number of hybridized DNA oligos, and therefore the signal brightness, and the usually high copy number of sRNAs in the small volume of a bacterial cell (Fig. 1).

Véronique Arluison and Claudio Valverde (eds.), *Bacterial Regulatory RNA: Methods and Protocols*, Methods in Molecular Biology, vol. 1737, https://doi.org/10.1007/978-1-4939-7634-8_12,

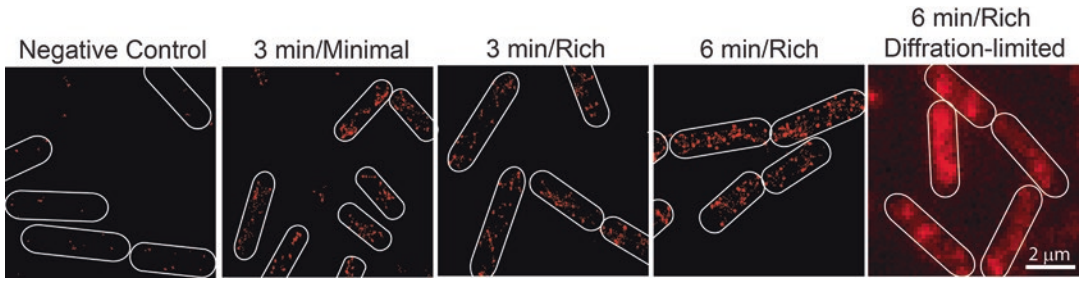

Fig. 1 Presentative images of SgrS. From the left to right are: negative control for probe nonspecific binding with Δ*sgrS* strain by SR imaging, 3 min post-induction by αMG in MOPS minimal medium by SR imaging, 3 min post-induction by αMG in MOPS rich medium by SR imaging, 6 min post-induction by αMG in MOPS rich medium by SR imaging, 6 min post-induction by αMG in MOPS rich medium by diffraction-limited microscopy. Cell boundaries are shown with white lines

Here, we provide a protocol of combining smFISH with super-resolved single-fluorophore microscopy (such as stochastic optical reconstruction microscopy (STORM), photoactivated localization microscopy (PALM), and fluorescence photoactivation localization microscopy (FPALM), and hereafter referred as SR microscopy) [6–9], and determination of the copy number of sRNA in individual bacterial cells with clustering analysis [10]. In SR imaging, photo-switchable fluorophores are used to label the sample of interest such that at any given time, only a subset and sparsely localized fluorophores are activated into the bright state and the centroids of these fluorophores are pinpointed with nanometer accuracy [7]. Repetitive activation and imaging allow reconstruction of final images with about a 10-fold enhancement in spatial resolution compared to the diffraction-limited microscopy. A density-based clustering analysis algorithm, DBSCAN [10], is then applied to 3D reconstructed SR images to segregate individual spots into clusters, which allows further determination of RNA copy number.

We use an sRNA, SgrS, which is involved in glucose-phosphate stress in *E. coli* [11–13], as an example to illustrate the method. The same method can be used to quantify mRNAs [13]. We will include bacterial sample preparation, SR imaging, and copy number calculation in the protocol.

2 Materials

2.1 Bacterial Culture

1. MOPS rich defined medium: Solution is made using TEKnova® MOPS EZ Rich Medium Kit. For 500 mL of medium, mix 50 mL of 10× MOPS mixture, 5 mL of K_2HPO_4, 50 mL of 10× ACGU, 100 mL of 5× Supplement EZ, and 295 mL of water. Medium is sterilized using 0.2 μm filter. Store in the dark at room temperature.

2. MOPS minimal medium: For 500 mL of medium, mix 50 mL of 10× MOPS mixture and 5 mL of K_2HPO_4 and 445 mL of water. Medium is sterilized using 0.2 μm filter. Store in the dark at room temperature.

Carbon sources are added depending on the experimental needs. In our example case, 0.2% glucose and 0.2% fructose are mixed with MOPS rich or minimal media.

2.2 Labeling of FISH Probe

1. 1 M $NaHCO_3$, pH 8.6: Dissolve 4.2 g of $NaHCO_3$ in 40 mL of water. Adjust the pH with 5 M NaOH, and adjust the final volume to 50 mL with water.
2. Absolute ethanol.
3. 3 M NaOAc, pH 5: Dissolve 12.3 g of NaOAc in 40 mL of water. Adjust the pH with glacial acetic acid, and adjust the final volume to 50 mL with water.
4. Silicone-based purification columns, such as Bio-Spin P-6 column (Bio-Rad™) or G25 column (GE Healthcare Life Sciences™).

2.3 Fixation and Hybridization

1. 37% formaldehyde.
2. Deionized formamide.
3. Phosphorus-buffered saline (1×), pH 7.4, PBS.
4. Saline-sodium citrate (20×), SSC.
5. FISH wash buffer: 10% formamide in 2× SSC. Add 5 mL of 20× SSC and 5 mL of deionized formamide to 40 mL of water. Store at 4 °C.
6. FISH hybridization buffer: 10% dextran sulfate and 10% formamide in 2× SSC. Weigh out 1 g of dextran sulfate (average molecular weight >500,000) and add to a 15 mL conical tube. Add 1 mL of 20× SSC, 1 mL of formamide, and 7 mL of water to the tube. Incubate at room temperature with shaking or vortexing until the dextran sulfate dissolves. Adjust the final volume to 10 mL with water. Divide the solution into aliquots and store at −20 °C.

2.4 Microscope

SR microscopy setup can be configured in many ways, and here we describe our example (Fig. 2). An inverted optical microscope (Nikon Ti-E with 100× NA 1.49 CFI HP TIRF oil immersion objective) can be combined with laser illumination, such as a red laser (647 nm, 120 mW, Cobolt MLD) and a violet laser (405 nm, 25 mW, CrystaLaser), through fiber coupling. Laser excitation lights are reflected by a dichroic mirror (Chroma zt405/488/561/647/752rpc-UF3) for near-TIRF excitation. The emission signal is collected by the objective lens, filtered by the emission filter (Chroma ET700/75 m), and imaged by a

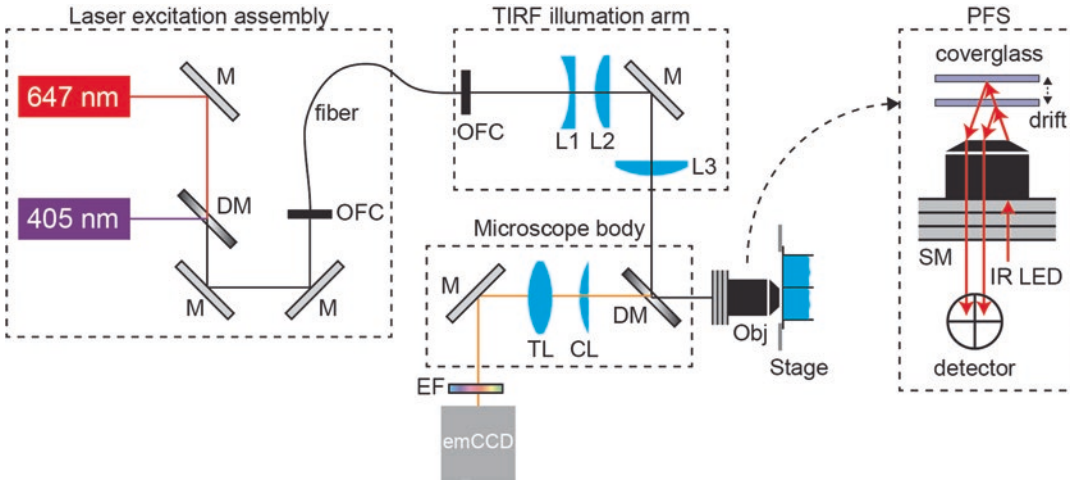

Fig. 2 Schematic illustration of the microscope setup. *M* mirror, *DM* dichroic mirror, *OFC* optical fiber coupling, *L1 & L2* lenses for expanding the beam, *L3* lens at the back focal plane, *Obj* objective lens, *CL* cylindrical lens, *TL* tube lens, *EF* emission filter, *SM* step motor. *PFS* Perfect focus system from Nikon, utilizing near-infrared 870-nanometer LED and CCD line sensor, is used to maintain the *z* stability

1024 × 1024 pixels EMCCD camera (Andor iXon Ultra 888). For 3D imaging, a cylindrical lens with 10 m focal length (CVI RCX-25.4-50.8-5000.0-C-415-700) is inserted in the emission path to cause the astigmatism effect of fluorophores. Single molecule samples (labeled DNAs or proteins immobilized on a coverslip) can be imaged in different *z*-planes to correlate the astigmatism effect with the corresponding *z* values. Then by interpolation and fitting, a calibration curve is created to obtain *z* values of single fluorophores in actual imaging [7]. To maintain the *z*-focus while imaging, Nikon Perfect Focus System (PFS) is used. The laser power density on the sample is about 2000 W cm^{-2} for the red laser, and the maximum power density for the violet laser is about 130 W cm^{-2}.

2.5 Imaging

1. 2-Metacaptoethanol (BME, Sigma-Aldrich M6250).
2. Catalase (EMD Millipore 219001): Make aliquots of 454.5 kU/mL =33.6 mg/mL in 50% glycerol/1× PBS and keep them in −20 °C.
3. Glucose oxidase (Sigma-Aldrich G2133): Make aliquots of 100 mg/mL (>10 KU/mL) in 50% glycerol/1× PBS and keep them in −20 °C.
4. The imaging buffer: 10 mM NaCl, 50 mM Tris and 10% glucose in 1× PBS (for immunostained samples) or 4× SSC (for FISH samples), with pH adjusted to 8.
5. 8-Well chambered cover glass (Nunc Lab-Tek 155409 or Cellvis C8-1.5H-N).
6. Poly-L-lysine (Sigma Aldrich P8920).
7. Nikon NIS-Elements software for image acquisition.

2.6 Image Processing and Data Analysis

1. IDL for reconstruction of 3D SR images.
2. MATLAB for copy number analysis.

3 Methods

3.1 FISH Probe Design, Labeling, and Purification

1. FISH probes are designed using Stellaris® Probe Designer 4.2 (*see* **Note 1**). Probes contain an amine modification for fluorophore conjugation.
2. The following protocol for probe labeling is for a total reaction volume of 15 μL. Add 1.5 μL of 1 M $NaHCO_3$ (pH 8.6) to 13.0 μL 100 μM amine-modified DNA oligo and mix. In a separate tube, dissolve 0.025 mg of fluorophore in 0.5 μL DMSO. Mix the dye solution with the oligo solution and incubate at 37 °C overnight in the dark (*see* **Note 2**).
3. On the next day, purify the labeled DNA oligos from the free dyes with ethanol precipitation. Add 1/9 reaction volumes of 3 M NaOAc (pH 5) and 2.5 volumes (of total volume including reaction and NaOAc solution) of 100% ethanol to the labeling reaction. Incubate for at least 6 h at −20 °C in the dark. Centrifuge the probe solution at full speed (~21,000 × *g*) for 30 min and then resuspend the pellet in 30–40 μL of water.
4. Further purify the labeled oligo from the residual free dyes with silica-based purification column, according to the manufacturer's instruction.
5. Take the UV-Vis absorption spectrum of the sample to calculate the concentrations of the DNA oligo, the fluorophore, and the labeling efficiency (defined by the ratio of the concentration of the fluorophore to the concentration of the oligo).

3.2 Culture Preparation, Fixation, and Hybridization

SgrS is used as an example here for the demonstration of the imaging and copy number calculation. Along with the wild-type strain, a *ΔsgrS* strain is also prepared as a negative control. SgrS is induced with methyl α-D-glucoside (αMG). A sample containing a relatively low-copy number and well-separated SgrS is prepared for RNA copy number calculation (referred as "single-RNA calibration sample," and *see* Subheading 3.5). In this case, we use SgrS induced for 3 min in MOPS minimal medium as the single-RNA calibration sample. Samples with SgrS induced for 3 min and 6 min in MOPS rich medium are prepared and analyzed as additional examples for comparison (Fig. 1).

1. Grow bacteria strains overnight in MOPS EZ rich medium (Subheading 2.1) supplemented with 0.2% glucose and 0.2% fructose at 37 °C at 250 rpm in a shaker. On the next day, dilute the overnight cultures 100-fold using fresh medium:

10 mL culture of *ΔsgrS* strain in MOPS EZ rich medium, 10 mL culture of wild-type strain in MOPS minimal medium, and 20 mL culture of wild-type strain in MOPS EZ rich medium. In each case supply the carbon source as 0.2% glucose and 0.2% fructose. Cells are grown again at 37 °C at 250 rpm until OD_{600} reaches 0.2–0.3 for SgrS induction.

2. Add 50% αMG to the cultures to a final concentration of 0.5% to induce SgrS. Harvest the cells (10 mL each) at different time points post induction by directly adding 1.2 mL 37% formaldehyde to the culture (final concentration of 4%) to fix the cells.
3. Cells mixed with formaldehyde are incubated for 30 min on a nutator at room temperature.
4. Collect the cells by centrifuging at 4000 × *g* for 10 min at room temperature.
5. Wash cells twice in the reaction volume of 1× PBS by resuspending, centrifuging at 600 × *g* for 3.5 min, and removing the supernatant. The reaction volume is calculated such that the density of the cells is ~5×10^9 per mL (*see* **Note 3**).

$$\text{Reaction volume}(\text{mL}) = (\text{Number of cells from the culture}) / 5 \times 10^9$$

6. Permeabilize cells by resuspending the pellet in the reaction volume of 70% ethanol. To avoid clumping of cells, first resuspend cells in water and then mix with 100% ethanol to a final concentration of 70% ethanol. Incubate cells in 70% ethanol for at least 1 h at room temperature on a nutator (*see* **Note 4**).
7. For hybridization, take 60 μL of cells in 70% ethanol. Centrifuge for 7 min at 600 × *g* and remove supernatant. Resuspend with 100 μL of 10% FISH wash solution and leave for a few minutes.
8. Prepare the hybridization mix in a fresh tube. Mix the labeled oligos with the appropriate amount of FISH hybridization buffer. The final concentration is 50 nM per probe for sRNA and 15 nM per probe for mRNA.
9. Centrifuge cells for 7 min at 600 × *g* and remove supernatant. Add 15 μL of hybridization mix to each cell sample and mix, avoiding air bubbles. Incubate at 30 °C overnight.
10. On the next day, add 200 μL of FISH wash buffer to each hybridization reaction, mix, centrifuge at 600 × *g* for 7 min, and remove supernatant.
11. Resuspend the pellet with 200 μL of FISH wash buffer and incubate for 30 min at 30 °C. Afterward, centrifuge and remove supernatant. Repeat the wash step two more times. Resuspend the final cell pellet in 20 μL of 4 × SSC (*see* **Notes 5** and **6**).

12. To immobilize the cells, an 8-well chambered cover glass is coated with poly-L-lysine for 45 min, then washed with water three times and air-dried. Per each well, 5 μL of the cell resuspension from **Step 11** are diluted with 125 μL of 4× SSC and incubated in each well for >1 h in 4 °C for immobilization.

3.3 Imaging

1. Imaging buffer is mixed with oxygen scavenger system right before imaging. Add 5 μL of BME, 3 μL of Glucose oxidase, and 1 μL of catalase to 491 μL of the imaging buffer, and transfer the mixed buffer to the imaging well. Incubate the sample in the imaging buffer with oxygen scavenger and BME for a few minutes (*see* **Note 7**).
2. A DIC image is taken for an area of interest. Then count the number of cells in the imaging area (*see* **Note 8**).
3. Imaging acquisition is done with repetitive cycles with the sequence of violet (405 nm, 1 frame) → red (647 nm, 3 frames) as a single cycle. The imaging acquisition starts with 0 $W \cdot cm^{-2}$ violet laser power. The violet laser power is modulated so that the number of "blinking-on spots" is kept above 50% of the number of cells in the field of view. When the number of "blinking-on" spots reaches less than this, even with the maximum violet laser power (130 $W \cdot cm^{-2}$), the acquisition is terminated. The controlled and automatic acquisition is coded in the Jobs module in NIS-Element.

3.4 Imaging Reconstruction

The data analysis is based on a previously published algorithm [7, 14]. An analysis package coded in IDL is used for reconstruction of 3D SR images.

1. Peak Identification and Fitting: To remove the noise, the image is blurred by Gaussian convolution of 9 × 9 pixels.
   (a) A user-defined intensity threshold (usually ~4) is introduced. Then the software finds all the pixels whose values are greater than "threshold" × "standard deviation of all the pixel values of the entire frame."
   (b) The software finds the local maximum intensity pixels whose pixel values are greater than their 24 surrounding pixels.
   (c) The software removes overlapping spots or bright junks by applying sharpness (ranging from 0.4 to 3) filter. Sharpness is defined as the intensity ratio between the peak and the background.
   (d) For peaks filtered so far, the area of 19 × 19 pixels surrounding local maximum intensity pixel is fitted with an Elliptical Gaussian function,

$$G(x,y) = h\exp\left(-2\frac{(x-x_0)^2}{w_x^2} - 2\frac{(y-y_0)^2}{w_y^2}\right) + b$$

where (x_0, y_0) is the position of the peak, w_x and w_y are its widths, and h and b are remaining fitting constants.

2. Horizontal Drift Correction: Drift Correction by fast Fourier transformation. The dataset is divided into an equal number of frames, and sub-SR images appearing similar to each other are obtained, except for the effect of drift. Then the fast Fourier transformation (FFT) is applied to each sub-SR image. By comparing the center images from each transformed image, the software determines the relative drift among sub-SR images. By linear interpolation, the drift is corrected through all the frames (*see* **Note 9**).

3.5 Copy Number Analysis

Copy number calculation is performed with analysis package coded in MATLAB. The package is free to distribute upon request.

1. Clustering analysis: Detecting clusters from the raw data is necessary for cleaning the nonspecific background of the raw data, as well as defining each cluster resulting from single or multiple RNAs. The detected clusters are then used for RNA copy number calculation. We use DBSCAN [10], a density-based algorithm, for cluster detection (Fig. 3). DBSCAN requires two input parameters as Npts and Eps (*see* **Note 10**). A "core spot" is defined if there are more than Npts spots (including itself) around it within Eps distance. As an example, Fig. 3A (upper penal) shows a case in which Npts = 7, hereby starting with two core spots, colored as red and blue. Any spot within the Eps distance of a core spot becomes a new core spot if more than Npts spots can be found within Eps distance around it, and becomes a part of the initial cluster. In this way, clusters can expand (Fig. 3A, the center panel). On the other hand, any spot within Eps distance from a core point becomes a "border spot," if it is surrounded by fewer spots than Npts within Eps distance. In this way, the cluster stops expansion (Fig. 3A, the lower panel). Spots not belonging to any cluster are considered background noise and are not considered further for the following analysis (Fig. 3A, the black spots). After the cluster analysis, we obtain the following information: (1) the total number of clusters in each cell, (2) the number of spots in each identified cluster, (3) the total number of spots in clusters in each cell, and (4) the average "radius" per each cluster. The radius of a cluster is defined as the average distance between the cluster center (the average of all the coordinates of spots in the cluster) and all the spots in the cluster.
2. Baseline correction: DBSCAN analysis gives the total number of "clustered" spots in each cell, but not all those spots come from the real RNAs. Even with the sequence-specific probes to label the RNAs of interest, nonspecific binding of probes yields

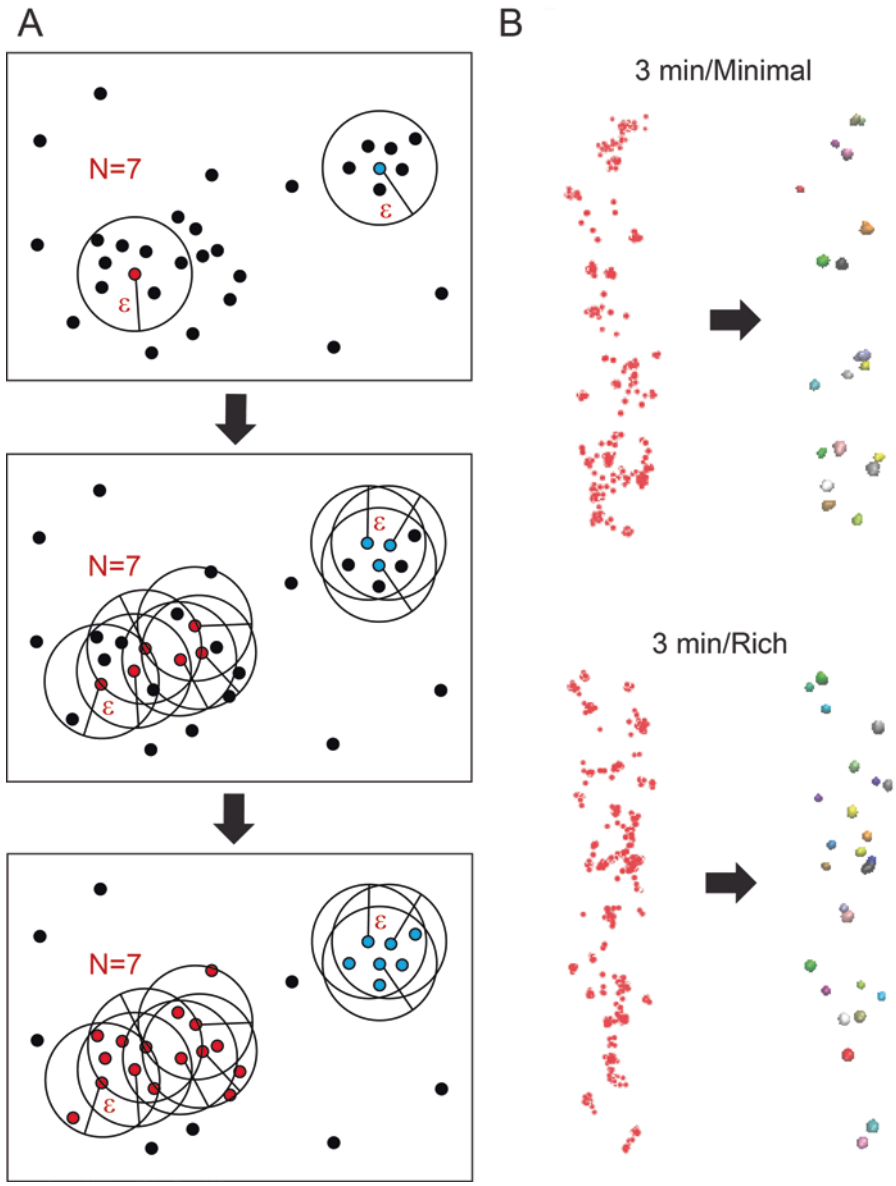

Fig. 3 Clustering analysis. (**A**) Illustration of DBSCAN algorithm. (**B**) Examples of DBSCAN on individual cells. Left: raw data from SR imaging; right: clustered data from DBSCAN, with each color representing each cluster

the false positive signal (the background signal) in each cell. Therefore, the tested samples all need to be corrected for the contributions from the background signal.

(a) Building a baseline for background signal: This background varies in different cells, as shown in the case of Δ*sgrS* cells labeled by SgrS FISH probes (Fig. 1, left most

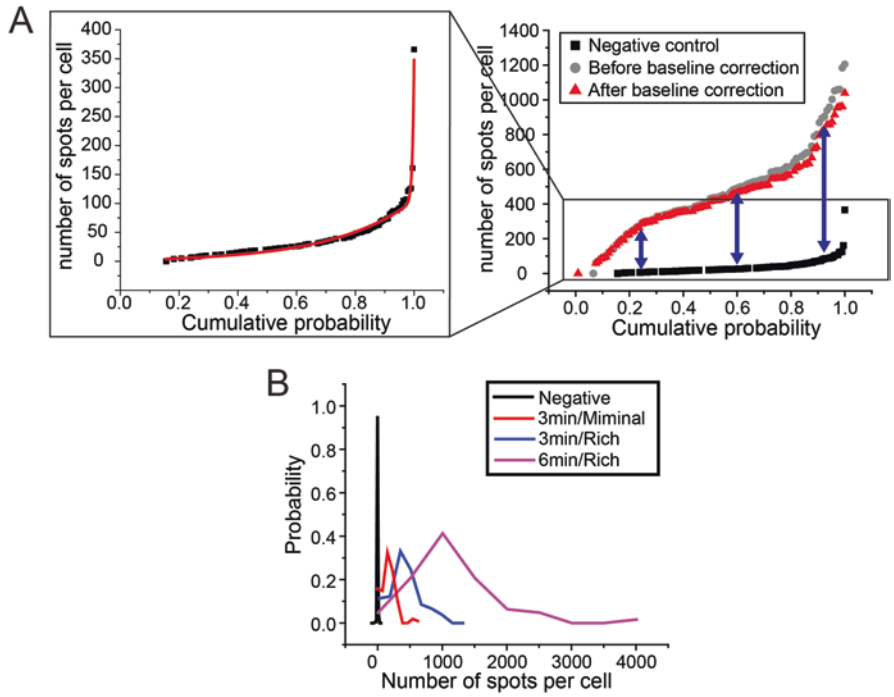

Fig. 4 Baseline subtraction for nonspecific binding. (**A**) *x–y* Inversed cumulative probability distribution of number of spots per cell in negative control is fit with double-exponential growth, which serves as a baseline for subtracting signal from probe nonspecific binding. (**B**) Distribution of the number of spots per cell after baseline subtraction

panel). We plot the cumulative probability distribution of background signal in individual cells by sorting the number of spots per cell from least to most (Fig. 4A, black points), and determine a baseline function by fitting this cumulative probability curve with a double exponential function (Fig. 4A, the red curve).

$$N_B = B + A_1 \exp\left(E_1 x - C_1\right) + A_2 \exp\left(E_2 x - C_2\right)$$

where x is the cumulative probability and N_B is the number of spots per cell. This function needs to be updated based on one's own negative strain collected with the SR microscopy setup.

(b) Baseline subtraction: The number of spots in each cell of the tested samples are sorted the same way, and subtracted by the baseline level of N_B calculated from the fitting curve obtained from the **step** (**a**) (Fig. 4B). Then the baseline-corrected number of spots per cells are used for the downstream analysis (Fig. 4C).

3. Single RNA characterization: This step requires single-RNA calibration sample, i.e., cells with low copy number and well-separated (not granule or transcription site forming) of

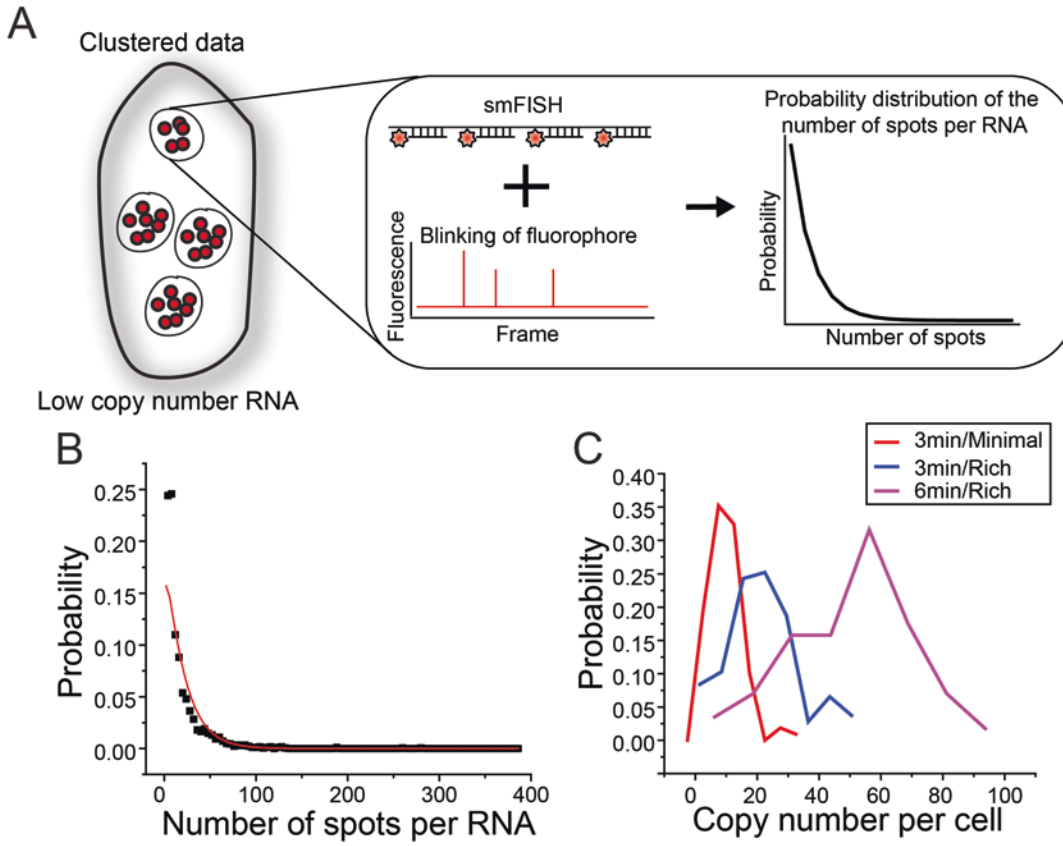

Fig. 5 Copy number calculation. (**A**) Illustration of copy number calculation. Single-RNA calibration sample is used to estimate number of spots per RNA, in which individual clusters are approximated as individual RNAs. The heterogeneity in the number of spots per RNA (cluster) is contributed by both the heterogeneities of number of hybridized probes per RNA and the number of spots (blinking events) generated per fluorophore. The resulting distribution of the number of spots per RNA (cluster) is empirically described by a negative binomial distribution. (**B**) Probability distribution of the number of spots per RNA from single-RNA calibration sample. (**C**) RNA copy number distributions in the three cases in Fig. 1

RNAs (Fig. 5A), such as cells fixed a short time after sRNA induction (Figs. 1 and 3 min/Minimal). In this case, we assume that DBSCAN analysis generates each distinct cluster corresponding to each RNA, and thus the number of spots per cluster represents the number of spots per RNA (N_0). We empirically fit the probability distribution of N_0 with a negative binomial distribution. This fitting is based on the assumption that each blinking event from each RNA is a Bernoulli trial, with the "ON" probability p, and we conduct imaging until r number of "OFF" states occur. We conduct imaging for a sufficiently long time so that the final event is always "OFF," as photobleaching. Then the number of "ON" states, i.e., the number of recorded spots per RNA (N_0) is fitted by the negative binomial distribution (Fig. 5A, B):

$$P(N_0;r,p)=\binom{N_0+r-1}{N_0}(1-p)^r\,p^r$$

These N_0 values are the observables which we extract from DBSCAN analysis for multiple clusters and from multiple cells, and by fitting this data we obtain p and r values (Fig. 5B).

4. Generation of $P(N|C)$ matrix: To obtain the number of RNAs in the cases of high-expression level, or granule forming, we utilize the convolution of negative binomial distribution. When there are RNAs with a copy number of C in a cell, given the distribution of N_0 per RNA, we can expect the distribution of a total number of spots (N) given the copy number of C to be:

$$P(N|C)=P(N;r^*C,p)=\binom{N+Cr-1}{N}(1-p)^{Cr}\,p^r$$

Based on this, we simulate a matrix of $P(N|C)$:

$$\tilde{P}(n|c)=\begin{bmatrix} P(N'=1|C'=1) & \cdots & P(N'=1|C'=c) \\ \vdots & \ddots & \vdots \\ P(N'=n|C'=1) & \cdots & P(N'=n|C'=c) \end{bmatrix}$$

5. Copy number calculation: According to Bayes' theorem and the law of total probability, probability of cells with N spots having RNA copy number of C can be calculated by:

$$P(C|N)=\frac{P(N|C)P(C)}{P(N)}=\frac{P(N|C)}{\sum_{C'}P(N|C')P(C')}P(C)$$

Assume $P(C')$ is uniform for all possible copy numbers, i.e., P(C′) = P(C),

$$P(C|N)=\frac{P(N|C)}{\sum_{C'}P(N|C')}$$

Then the expectation value of the RNA copy number C under the condition of observing N spots per cell is

$$E(C|N)=\sum_{C'}C'P(C'|N')=\frac{\sum_{C'}P(N|C')C'}{\sum_{C'}P(N|C')}$$

here $P(N|C')$ is obtainable from the constructed probability mass function matrix, $\tilde{P}(n|c)$ from **step 4**. Fig. 5C shows the distribution of RNA copy number per cell for multiple cells in different conditions in SgrS induction (also refer to Fig. 1).

4 Notes

1. Stellaris® Probe Designer 4.2 is a free program that designs probes based on the nucleotide sequence provided by the user. Maximizing the number of probes against a target increases the signal-to-noise ratio during imaging. Make sure that probes are not targeted to sequences involved in stable secondary structures.
2. The reaction can be scaled up when needed. In general, the amount of fluorophore used should be 25–30-fold in excess of the molar concentration of oligos. The exposure of fluorophore to room temperature should be minimized. Wrapping the tube with aluminum foil helps minimize its exposure to light.
3. The number of cells is estimated from OD_{600}. OD_{600} of 1 corresponds to a cell density of 3×10^8–1×10^9 depending on specific spectrometers.
4. Cells in 70% ethanol can be stored at 4 °C for a few weeks.
5. It is optional to perform postfixation; however, it can help preserve the hybridization of probes for longer. For postfixation, wash the cell sample with 200 μL of 1× PBS. Resuspend the pellet in 100 μL of 4% formaldehyde in 1× PBS and incubate for 10 min at room temperature on a nutator. Afterward, centrifuge, remove supernatant, and perform one more wash with 200 μL of 1× PBS.
6. It is highly recommended to image the cells when they are fresh to prevent signal loss due to probe dissociation.
7. Since pH will change over time due to the activity of glucose oxidase, imaging has to be done within ~ 1 h.
8. Cell counting can be done either by eye or by commercial software, such as Nikon NIS-Element's Analysis Explorer → "Cell count" feature.
9. An alternative way to correct the drift is to track the position of a fiducial marker, such as a nano-diamond, during data acquisition.
10. The choices of Npts and Eps are empirical depending on the specific microscopic setup, number of probes per RNA, fluorophore, and imaging conditions. It is recommended to try different combinations of Npts and Eps, and visually validate the clustered data compared to raw data. In addition, experimental validation, such as quantitative PCR, is recommended to compare with the copy number calculation from imaging approach.

Acknowledgments

The data presented as examples were collected during the 2016 Center for the Physics of Living Cells (CPLC) Summer School at University of Illinois at Urbana-Champaign. We therefore thank National Science Foundation grant PHY-1430124 (Physics Frontiers Center for the Physics of Living Cells) for funding for the CPLC Summer School, Zan Luthey-Schulten and Joseph R. Peterson for co-teaching the scientific theme of "Quantitative Imaging and Cell Simulation of Small Regulatory RNA," and the summer school students: Bijoy Desai (Columbia University), Dennis Fernandes (University of Toronto, Mississauga), Jialei Tang (University of Central Florida), and Kailun Zhang (Texas A&M University) for participation in data collection and analysis.

References

1. Wagner EGH, Romby P (2015) Small RNAs in bacteria and archaea: who they are, what they do, and how they do it. Adv Genet 90:133–208
2. Storz G, Vogel J, Wassarman KM (2011) Regulation by small RNAs in bacteria: expanding frontiers. Mol Cell 43:880–891
3. Femino AM, Fay FS, Fogarty K, Singer RH (1998) Visualization of single RNA transcripts in situ. Science 280:585–590
4. Raj A, van den Bogaard P, Rifkin SA, van Oudenaarden A, Tyagi S (2008) Imaging individual mRNA molecules using multiple singly labeled probes. Nat Methods 5:877–879
5. So L-H, Ghosh A, Zong C, Sepúlveda LA, Segev R, Golding I (2011) General properties of transcriptional time series in Escherichia coli. Nat Genet 43:554–560
6. Betzig E, Patterson GH, Sougrat R, Lindwasser OW, Olenych S, Bonifacino JS, Davidson MW, Lippincott-Schwartz J, Hess HF (2006) Imaging intracellular fluorescent proteins at nanometer resolution. Science 313:1642–1645
7. Huang B, Wang W, Bates M, Zhuang X (2008) Three-dimensional super-resolution imaging by stochastic optical reconstruction microscopy. Science 319:810–813
8. Rust MJ, Bates M, Zhuang X (2006) Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). Nat Methods 3:793–795
9. Hess ST, Girirajan TPK, Mason MD (2006) Ultra-high resolution imaging by fluorescence photoactivation localization microscopy. Biophys J 91:4258–4272
10. Daszykowski M, Walczak B, Massart D (2001) Looking for natural patterns in data. Chemom Intell Lab Syst 56:83–92
11. Vanderpool CK, Gottesman S (2004) Involvement of a novel transcriptional activator and small RNA in post-transcriptional regulation of the glucose phosphoenolpyruvate phosphotransferase system. Mol Microbiol 54:1076–1089
12. Rice JB, Vanderpool CK (2011) The small RNA SgrS controls sugar-phosphate accumulation by regulating multiple PTS genes. Nucleic Acids Res 39:3806–3819
13. Fei J, Singh D, Zhang Q, Park S, Balasubramanian D, Golding I, Vanderpool CK, Ha T (2015) RNA biochemistry. Determination of in vivo target search kinetics of regulatory noncoding RNA. Science 347:1371–1374
14. Bates M, Blosser TR, Zhuang X (2005) Short-range spectroscopic ruler based on a single-molecule optical switch. Phys Rev Lett 94:108101