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Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see our Editorial Policies and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒	A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒	The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☒	☐	A description of all covariates tested
☒	☐	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒	A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☒	☐	For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	Custom journals were written in Metamorph 7.8.13.0 software to acquire time-stream single-molecule images.
Data analysis	1. CRISPR design: ChopChop webtool (https://chopchop.cbu.uib.no/) 2. Routine image processing (like making montage, adding scale bar, changing lookup table, creating Kymographs, etc): Fiji (ImageJ 1.52p). 3. Single molecule localization: ThunderSTORM(dev-2016-09-10-b1). All the parameters used for ThunderSTORM can be found in (https://github.com/XiaoLabJHU/SMT_Unwrapping). 4. Single molecule tracking and post processing methods were done with custom programming codes written in Matlab (2020a) for spots linking and velocity analysis. The nearest neighbor algorithm was adopted from (Sbalzarini, I. F. & Koumoutsakos, P. Feature point tracking and trajectory analysis for video imaging in cell biology. J Struct Biol 151, 182–195 (2005).) See Methods section of manuscript for more details. Codes are available (https://github.com/XiaoLabJHU/SMT_Unwrapping).

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Key data to support the conclusions of the manuscript are shown in the main figures and Extended Data figures (Source Data). Additional substantiating data, including raw data for all figures, are available upon request.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	No specific sample size calculation or statistic method was made. The sample size varied in different experiments described below and in the manuscript. For cell wall labeling experiment, at least 600 E. coli cells were imaged and analyzed from three biological independent experiments, which is robust to ensure the standard error of mean small enough to compare the average labeling levels. For growth curve measurements and immunoblots, two to three repeats were used to get the average results. For single molecule tracking experiments, at least 200 trajectories (the numbers are listed in the Supplementary tables) were monitored to reconstruct the distribution and cumulative probability density function for fitting. The errors of mean were clearly listed in the main text and supplementary tables.
Data exclusions	1. The fluorescence spots with intensity over 2 sigma of the peak intensity are removed from analysis since they are likely to be multiple molecules, which ensured the observation was made on single FtsW/FtsI molecules. 2. The fluorescence spots on the edge of the cell(>0.75 radius from the center) were excluded. This is an empirical threshold used in this study to minimize the unwrapping error. 3.The short trajectories (shorter than 6 time points) of a single molecule are excluded, because they are not able to be classify or quantify to moving or free diffusion molecules based on our simulation. More detailed description can be found in the Supplementary discussion.
Replication	All biological replicates were done by culturing different E. coli colonies with the same genotype. The cell growth, drug treatment, and imaging experiments were taken at different days with other conditions (such as temperature, cell density, etc) as the same as possible. The variation among replicates was describes in the text or figure legends.
Randomization	Samples were not allocated to groups.
Blinding	For the cell wall labeling experiment, another author reanalyzed the data partially blinded without know the counting result. For the single molecule tracking, the data collection is intrinsically blinded since the information from single molecules was randomly collected and not be able to choose with bias. Data analysis was not blinded considering the analysis pipepline is computer-based and standard.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☒	☐ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	anti-RFP (Thermo Fisher Scientific) Catalog # R10367; Anti-FtsN serum (From Dr. David S. Weiss);Goat Anti-Rabbit IgG (H + L)-HRP (Bio-Rad) Conjugate #1706515
Validation	anti-RFP: the validation information can be found on the website: https://www.thermofisher.com/order/genome-database/dataSheetPdf?producttype=antibody&productsubtype=antibody_primary&productId=R10367&version=123 specifically, it has been tested for TagRFP: "This antibody can be used to detect native RFP, RFP variants, and RFP fusion proteins in Western blot, immunoprecipitation, ELISA, and immunocytochemistry. This antibody has been shown to detect denatured and native TurboRFP, TurboFP602, TurboFP635, TagRFP, TagFP635, and mKate2" anti-FtsN has been published (Wissel M C, Weiss D S. Genetic analysis of the cell division protein FtsI (PBP3): amino acid substitutions that impair septal localization of FtsI and recruitment of FtsN[J]. Journal of bacteriology, 2004, 186(2): 490-502.)