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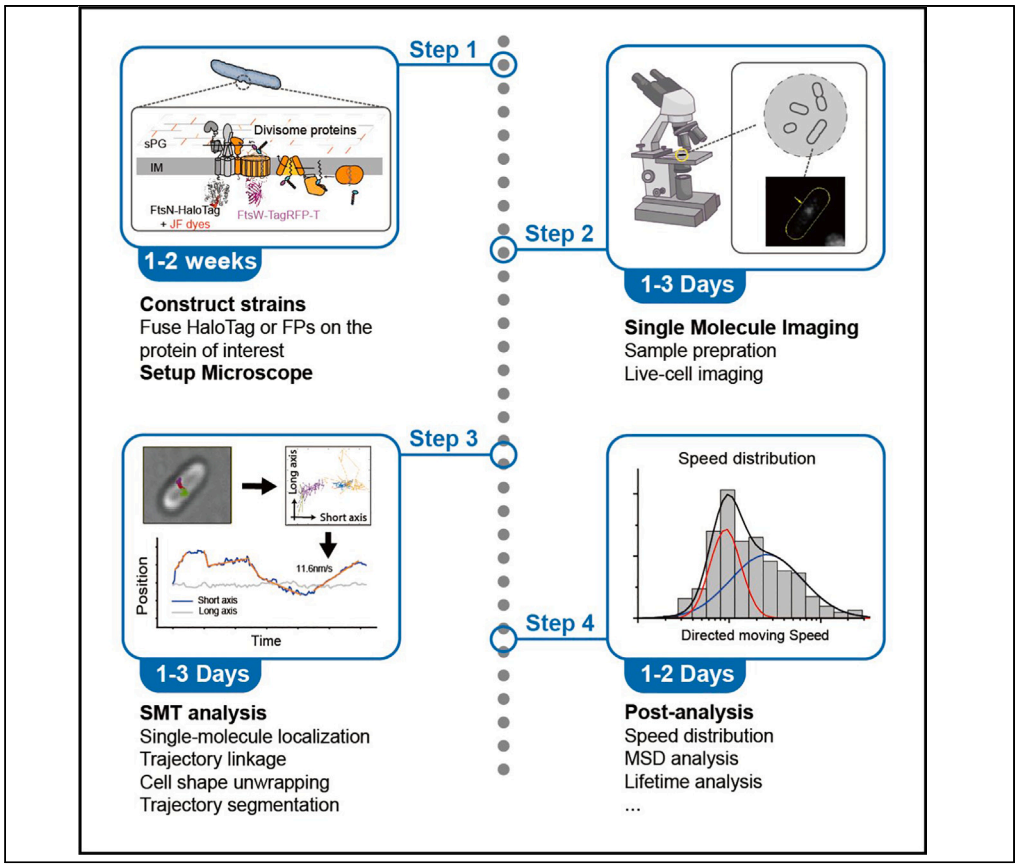
Protocol for single-molecule labeling and tracking of bacterial cell division proteins



Here, we present a protocol for labeling and tracking individual molecules, particularly cell division proteins in live bacterial cells. The protocol encompasses strain construction, single-molecule imaging, trajectory segmentation, and motion property analysis. The protocol enables the identification of distinctive motion states associated with different cell division proteins. Subsequent assessments of the dynamic behaviors of these proteins provide insights into their activities and interactions at the septum during cell division.

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

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Highlights

Real-time single-molecule tracking to monitor individual proteins in living bacterial cells

Accurate displacement calculation of membrane proteins via cell curvature correction

Identification of different motion states such as confined diffusion and directed movement

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Protocol for single-molecule labeling and tracking of bacterial cell division proteins

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SUMMARY

Here, we present a protocol for labeling and tracking individual molecules, particularly cell division proteins in live bacterial cells. The protocol encompasses strain construction, single-molecule imaging, trajectory segmentation, and motion property analysis. The protocol enables the identification of distinctive motion states associated with different cell division proteins. Subsequent assessments of the dynamic behaviors of these proteins provide insights into their activities and interactions at the septum during cell division.

For complete details on the use and execution of this protocol, please refer to Yang et al. (2021),[1] Lyu et al. (2022),[2] and Mahone et al. (2024).[3]

BEFORE YOU BEGIN

Walled bacterial cells employ more than thirty proteins to assemble into a macromolecular machinery, collectively termed ***divisome***, to perform the essential task of cell division.[4,5] FtsZ, a cytoskeletal protein, and a tubulin homolog, forms a ring-like structure at the division site and recruits other divisome proteins in a largely hierarchical order.[6] FtsN, a conserved sporulation-repeat-related-domain (SPOR) protein and the last essential divisome protein recruited in *E. coli*, also forms a ring-like structure.[2] The spatial organizations of both the Z- and N-ring have been investigated using super-resolution microscopy, revealing a patchy, heterogeneous organization for both rings.[2,7–10] The dynamics of some essential divisome proteins, including FtsZ,[11–13] FtsN,[2] FtsB,[14] and the sPG synthase complex FtsW/FtsI,[1,3,13,15–20] have been investigated using single-molecule tracking (SMT) in a few bacterial species. SMT provides real-time monitoring of a single molecule's positions and dynamic movements with high spatial-temporal resolutions in living bacterial cells, leading to the identification of various motion states of these divisome proteins. These distinct motion states were further associated with unique functions and interactions of these proteins. In contrast, ensemble fluorescence imaging and traditional biochemical approaches, such as co-immunoprecipitation (co-IP) and yeast two-hybrid (YTH) experiments, typically report on the average behaviors of bulk populations of molecules. SMT allows for differentiating sub-populations and heterogeneous molecular behaviors, complementing conventional techniques.

This protocol describes SMT experiments and data processing methods based on our previous SMT studies of FtsW, FtsI, and FtsN dynamics in living *E. coli*[1,2] and *C. crescentus*[3] cells. We include methods suitable for two-dimensional (2D)-[1,3] and three-dimensional (3D)-SMT[2] experiments. Using

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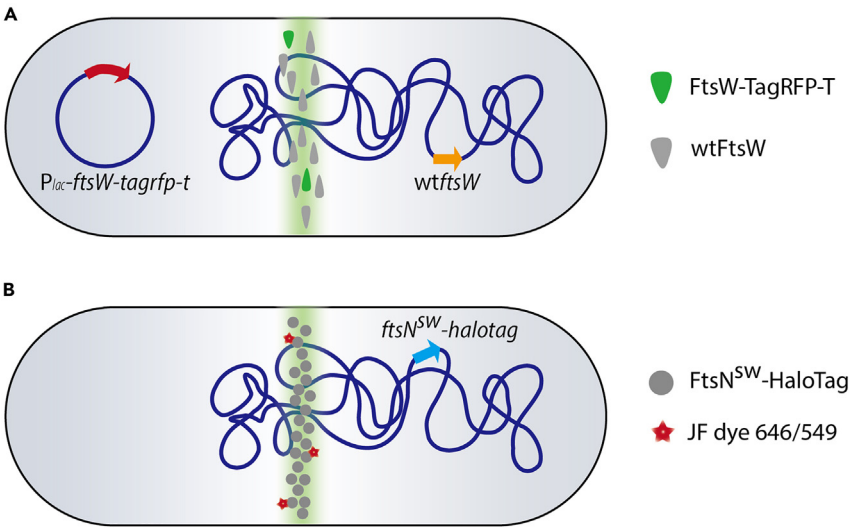

Figure 1. Scheme of FP or HaloTag fusion construct for single-molecule tracking

(A) The gene of an FP (e.g., *tagrfp-t*) is cloned to the downstream position of a division gene of interest (e.g., *ftsW*) on a plasmid. The expression level should be tightly controlled to ensure single-molecule detection.

(B) The gene encoding HaloTag is integrated into the *E. coli* chromosome to fuse with the native POI gene (e.g., *ftsN-halotag*). The labeling concentration and time of the dye (e.g., JF dye 646/549) should be adjusted to ensure single-molecule labeling.

a relatively long exposure time (0.5–1 s) for image acquisition, freely diffusing protein molecules on the membrane with a diffusion coefficient greater than 0.1 $\mu m^2/s$ are blurred out, only leaving in focus with molecules that are either moving slowly or stationary due to their association with divisome or peptidoglycan (PG). Multiple positions from a single molecule in the septum region are linked to generate raw single-molecule trajectories. These trajectories are "unwrapped" from the curved membrane plane due to the cylindrical cell shape to obtain their corrected coordinates on a flat surface. Subsequently, trajectories are segmented based on distinct motion states through statistical means to isolate directionally moving and stationary segments. From these segments, essential parameters, such as directional moving speeds, diffusion coefficients, confinement sizes of stationary states, and the lifetime of each state, can be quantified.[1,2] This protocol's imaging and analysis procedures can also be applied to study cell division proteins of different bacterial organisms or other proteins exhibiting directional motions.

When combined with genetic and biochemical manipulations, the moving dynamics of a divisome protein can provide further insight into its activities and interactions with other divisome components. For instance, by comparing the speed distributions of FtsW, FtsI, FtsB, and FtsN with the treadmilling speed of FtsZ, we found that cell wall synthases and regulators dynamically switch between two "tracks" to be activated or inactivated during cell wall constriction in *E. coli*.[1–3,21] Inactive sPG synthase complexes composed of FtsWIQLB follow the fast-moving "Z-track" to be distributed along the septum and become activated when they switch to the slow-moving "sPG-track" to form a processive complex with FtsN. The lifetime distribution of the fast-moving population of FtsI molecules confirmed the Brownian-ratchet mechanism in which cell wall synthase complexes track the shrinking end of FtsZ filaments.[16]

Construct fluorescent protein (FP) or HaloTag fusion

Timing: 3–7 days

SMT experiments require sparsely distributed fluorescent molecules in each cell for accurate single-molecule localization and tracking. Two strategies are commonly applied to achieve the low labeling

density of the protein of interest (POI). The first strategy is to ectopically express the POI tagged with a fluorescent protein (FP) (e.g., FtsW-TagRFP-T[1]) using a stringent promoter to minimize the expression level to ~1 fusion POI-FP molecule per cell on average (Figure 1A). The second strategy is to use a self-labeling POI-HaloTag fusion, in which the number of labeled POI molecules can be controlled by tuning the concentration and labeling time of the fluorescent ligand for HaloTag (Figure 1B). Both fusion genes can be expressed from a plasmid in the presence of the native gene copy (Figure 1A), or as the sole copy of the POI by replacing the endogenous copy (Figure 1B). The selection of the appropriate strategy depends on the experimental design and the ease of genetic manipulation for the target POI.[1,2] Most importantly, users should take time to verify the functionality of the POI fusion. In our studies, we routinely assess whether the fusion protein can rescue the null or depletion background of the native copy by examining the corresponding growth rate, cell morphology, and cell division phenotypes. This section describes the procedures for generating FP or HaloTag fusion constructs in *E. coli*.

Construct FP-fusion on plasmid vectors

In wild-type (WT) *E. coli* strains such as BW25113[22] or TB28,[23] we optimized two IPTG (Isopropyl β-D-1-thiogalactopyranoside)-inducible vectors for POI-FP (TagRFP-T[1]) expression (key resources table) to achieve an extremely low expression level. FP can be tagged to the N- or C-terminus of the POI via a short peptide linker or inserted at an appropriate site in the middle of the POI (sandwich fusion[2,16]). We used a flexible linker, GGGGSPAPAPGGGGS, for our studies. Readers may need to optimize the linker sequence and length to align with their research objectives.

To construct the fusion gene, the double-stranded DNA fragment of the POI gene, the FP gene containing the linker sequence, and the vector backbone are amplified with two flanking 15 bp overlap sequences on each end. These DNA fragments are then recombined together by In-fusion cloning (Takara, In-Fusion HD Cloning Kit). Two plasmids, pJB008 (TagRFP-T-FtsI) and pXY349 (FtsW-TagRFP-T), which offer slightly different expression levels, along with their respective maps, can be obtained from Addgene (#98995 and #200262, respectively).

> ***Note:*** To achieve a sufficiently low expression level of FP fusion proteins for SMT, we suppressed the basal expression of FP fusions using the $lacI^Q$ promoter (pJB008) with LacI expression from the same plasmid or by further modifying the −35 region of the $lacI^Q$ promoter (GTGCAA to TTGACA) using Quikchange Lightening Kit (pXY349).

Before imaging experiments, carefully examine cells that express the final fusion construct to ensure normal cell morphology and growth rates compared to WT cells. After cells reach the early log phase (OD_{600} = 0.1–0.2), induce the culture with 1–100 μM IPTG for ~2 h and image cells to verify the correct spatial distribution of the POI-FP in live cells. For single-molecule imaging, adjust IPTG concentration to ensure that only a few fluorescent spots are visible in each cell (Method in single-molecule imaging section). Notably, no additional IPTG is required for the FtsW/FtsI vector in our studies as the basal leaky expression level is sufficient.

POI-FP expressed from alternative expression systems should also work if a low expression level (sparse signals) and normal cell morphology are achieved.

Construct HaloTag-fusion on *E. coli* chromosome

To supply the fusion gene as the sole cellular source of POI, we integrate the fused gene into the native chromosomal locus, replacing the endogenous gene using a CRISPR-mediated λ-Red recombineering method.[1,16] This method allows us to insert the *halo* gene into the endogenous *ftsI* gene locus to construct a HaloTag-$FtsI^{SW}$ fusion *E. coli* strain.[16] Here, we describe another way of using the CRIM plasmid-host systems[24] to integrate a single copy of the *halo-ftsN*^{SW} gene into the *attΦ80* site of the *E. coli* genome. Both methods could be applied to other genes of interest with modification and optimization.

The designed *halo* fusion gene (e.g., *halo-ftsN*$^{sw\ 2}$) with a specific promoter sequence (e.g., P_{204_7A}[2]) is amplified and In-fusion cloned (Takara, In-Fusion HD Cloning Kit) into the CRIM vector,[24] yielding pDSW2083 (key resources table). The CRIM plasmid, containing the γ replication origin of R6K, relies on the trans-acting π protein (encoded by *pir*) for replication.[25] The PIR2 strain harbors the *pir* gene to maintain the CRIM plasmid. Premade chemical- or electro-competent *ftsN*-depletion cells (strain EC1908) were first transformed with the CRIM helper plasmid pAH123, which encodes all the requisite Int proteins for the integration of CRIM plasmid into the *attΦ*80 site of the *E. coli* genome,[24] generating strain EC4440. This strain was further prepared electro-competent using a standard protocol[26] and transformed with the CRIM vector carrying the *halotag* fusion gene for insertion at the *att* site. Cells were recovered at 37°C for 45 min, followed by an additional 45 min at 42°C to eliminate the helper plasmid.

Note: If the *halo* fusion gene is located at a different site on the chromosome, an inducible promoter is recommended to adjust the expression level. The P_{204_7A} promoter used in our study could be a starting point for optimization.[2] The native gene copy could be knocked out using a standard λ-red recombination protocol.[26] If the *halotag* fusion gene is the only source of the POI and cells can maintain the same phenotype as the WT strain, the POI-HaloTag fusion can be considered functionally equivalent to the WT protein.

Note: Whole-genome sequencing can be employed to verify no single nucleotide variants or insertion-deletion mutations, particularly in essential genes, were generated during the cloning process.

Preparation one: Prepare growth media

Timing: 1 h

This protocol requires the imaging medium to be consistent with the growth medium. Our *E. coli* SMT experiments primarily employ two distinct chemical-defined media. M9 is a minimal defined medium for a slow cell growth and division rate (approximately 150–180 min doubling time at 25°C). EZRDM is a rich, defined medium for relatively fast cell growth and division rate (about 40–60 min doubling time at 25°C). Complex media such as LB (Luria-Bertani broth) should not be used due to their high fluorescence background, which masks single molecule detection.

1. Preparation of M9 medium:
   a. Add M9 salts, MEM amino acids, Glucose, $MgSO_4$, and $CaCl_2$ sequentially to 75.8 mL of ddH_2O while stirring, following the recipe of M9 minimal medium.
   b. Sterilize the medium by filtration using Stericup Quick Release-GP Sterile Vacuum Filtration System.
   c. Store the medium at room temperature (between 18°C–27°C).

   Note: White precipitates may appear when adding $CaCl_2$. Keep stirring the solution for a couple of minutes until the precipitates disappear.

2. Preparation of EZ rich medium (EZRDM):
   a. Carefully aliquot the MOPS Modified Rich Buffer, ACGU Solution, and Supplement EZ from the MOPS EZ Rich Defined Medium Kit in 10, 10, and 20 mL, respectively. Store the aliquots at −20°C to avoid contamination.
   b. Thaw one aliquot of each component on bench.
   c. Mix the components according to the recipe of EZRDM with ddH_2O.
   d. Sterilize the medium by filtration using Stericup Quick Release-GP Sterile Vacuum Filtration System.
   e. Store the medium at 4°C.

   Note: EZRDM is prone to contamination. Prepare a small amount for each experiment each time.

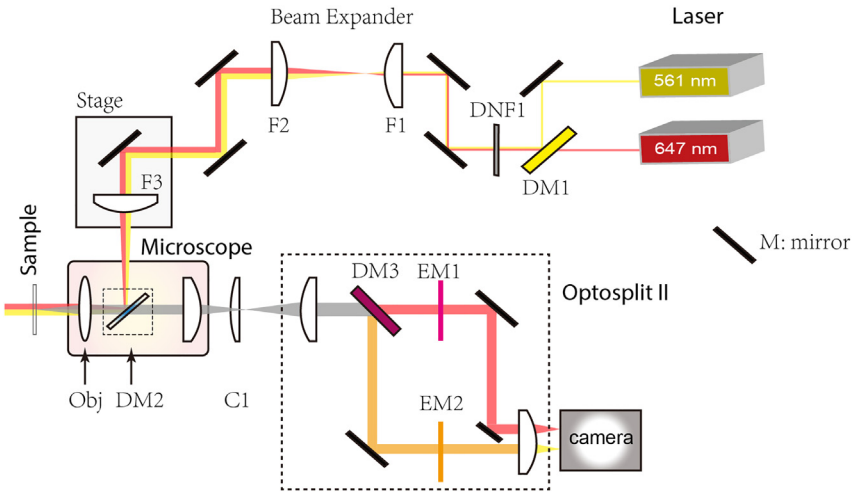

Figure 2. Schematic diagram of single-molecule tracking microscope setup
Detailed information for each component can be found in Table 1.

Preparation two: Prepare JF dye stocks

Timing: 1 h

3. Dissolve the JF646 or JFX650 Halo ligand in DMSO to a concentration of 100 μM.
4. Aliquot in small volumes (5–10 μL) to avoid repetitive freeze/thaw cycles. The aliquots are kept at −80°C and protected from light.
5. Dilute one aliquot to a final concentration of 1 μM in ddH_2O for the labeling experiments. The solution can be stored at −20°C for 2–3 weeks.

Preparation three: Clean cover glasses

Timing: 2 h

6. Arrange ten pieces of 40 mm round coverslips in an adjustable cover-glass rack and settle the rack in a clean glass container.
7. Make 1% Alconox and 1 M KOH in ddH_2O.
8. Sonicate the coverslips sequentially in 1% Alconox, ddH_2O, 99% Ethanol, 1 M KOH, and ddH_2O for 15 min. Rinse the whole container with the rack three times in ddH_2O between every sonication step.

Note: Store the cover glasses in ddH_2O. Before use, blow-dry them with filtered air.

Preparation four: Setup single-molecule microscope

Timing: 4–7 days

In this protocol, the optical setup for SMT is based on an inverted fluorescence microscope with a home-built optical path performing Highly Inclined and Laminated Optical sheet (HILO) illumination with an EMCCD camera to detect the signal of single fluorophores.[27] The system is similar to the one we described for PALM imaging[8] and is illustrated in Figure 2. A list of its components can be found in Table 1.

9. Place the lasers, microscope body, and other components on an optical table according to Figure 2.

Table 1. Components for the microscope setup

Optical components	Brief description	Source	Note
647 laser	As excitation light of near-infrared fluorescence probes such as Janelia Fluor JF646	Coherent OBIS 647LX 120 mW	Other CW lasers with a max power ~100 mW can also satisfy the requirement. The wavelength should match with the dichroic mirror (DM) used.
561 laser	As excitation light of TagRFP-T or other red fluorescence probes (i.e., JF549 and JF552)	Coherent Sapphire 561 LP 100 mW	The wavelength should match with the dichroic mirror (DM) used.
M	Mirrors	Thorlabs BB1-E02P	–
DM1	Dichroic mirror for combing the excitation lasers (561 nm and 647 nm)	Chroma T600lpxr	Other types of beam combiner can be used.
DM2	Dichroic mirror to reflect the excitation lasers and pass the fluorescence signal	Chroma ZET405/488/561/647m-TRF	Choose the dichroic beam splitter based on the spectrum of FP or dye used in the experiments.
DM3	Dichroic mirror to separate fluorescence signal with different colors	Chroma T647lpxr	Choose the dichroic beam splitter based on the spectrum of FP or dye used in the experiments.
DNF1	Neutral density filter to reduce the excitation power if needed	Thorlabs FW102CWNEB	Optional, depends on specific experiments.
F1	Achromatic convex lens, f = 50 mm	Thorlabs ACN254-050-A	The laser beam is expanded to f2/f1 fold in diameter.
F2	Achromatic convex lens, f = 150 mm	Thorlabs ACN254-150-A	
F3	Achromatic convex lens, f = 300 mm	Thorlabs ACN254-300-A	This lens is placed 300 mm far from the back focal plane of the objective.
Stage	One-dimensional motorized stage	Thorlabs XR50C	The stage is for moving the laser beam horizontally and generating Epi, Hilo, or TIRF illumination.
Microscope	With a motorized stage (for X-Y) and piezo stage (for Z)	Olympus IX73 or IX83	Other commercial microscopy such as Nikon Ti-2 is also adequate for this application.
Obj	High NA objective with a good point spread function and an adjustable ring to reduce spherical aberration	Olympus UPLAPO100XOHR	Other 60× to 100× plan apo objective with NA > 1.45 should also work for this application.
C1	Achromatic cylindrical lens, f = 700 mm	Thorlabs LJ1836L1-A	This lens is for astigmatism-based 3D single-molecule localization.
EM1	Emission filter for the near-infrared channel (~ 700 nm)	Chroma ET700/75m	–
EM2	Emission filter for the red channel (~600 nm)	Chroma ET600/70m	–
OptoSplit II	Commercial color splitter to separate fluorescence with different wavelength in space	Cairn Research OptoSplit II	Home-built or other commercial color splitters can also be used.
Camera	To image the single molecule signal	Andor iXon 897	Other back-illuminated EMCCD or sCMOS cameras are suitable for the application too.
Sample chamber	To sandwich bacterial cells and keep them alive for imaging on microscope	Bioptechs, FCS II system	Home-made chamber or sandwich the cells between the coverslip and an ultra-pure agar pad are also good if no temperature control is required.

*A certain number of additional parts are required to build the microscope depicted in Figure 2, such as lens mounts (Thorlabs, KM100), posts (Thorlabs, TR6), post holders (Thorlabs, PH6), base adapters (BE1), clamping forks (CF175C), and some alignment tools.

10. Place and adjust the angle of the Dichroic mirror (DM1) and mirror to combine the two laser beams.
11. Adjust the position of the lenses (F1 and F2) to expand the laser to collimated beams with ~10 mm diameter. Ensure the laser beam passes through the lens centers and is perpendicular to their surface. The distance between the two lenses is the sum of their focal lengths.
12. Place the stage and set the mirrors to guide the expanded laser beam into the back aperture of the microscope. Adjust the two mirrors to align the laser beam through the microscope's light path.
13. Place the tube lens F3 according to Figure 2. Adjust the position of F3 along the light path to minimize the laser spot size after the objective. The tube lens with a focal length of 300 mm focuses the laser beam to the back focal plane of the high numerical aperture (NA) objective.
14. For two-dimensional SMT, install the OptoSplit II (Cairn Research) and the camera at the C-mount of the microscope. The OptoSplit II should be adjusted according to the manufacturer's protocol (https://www.cairn-research.co.uk/wp-content/uploads/2021/01/Optosplit-II-Manual.pdf).

15. For three-dimensional SMT,[2] insert a cylindrical lens C1 with a focal length of 700 mm near the intermediate focal plane.

Note: Translate the stage laterally using a Micrometer to archive Epi, HILO, or TIRF illumination mode. HILO mode is preferred for a higher illumination depth to image the divisome, especially in constricted cells.

Note: Other platforms, including commercial SMLM systems such as N-STORM (Nikon) and Nanoimager (Oxford Nanoimaging Limited), as well as custom-built SMLM systems, are also suitable for SMT applications.

Preparation five: Setup computing platform

Timing: 1–2 h

16. Download and install **ImageJ**[28] or **Fiji**[29] from https://imagej.net/downloads.
17. Install the "ThunderSTORM" plugin following the guidelines on https://zitmen.github.io/thunderstorm/.[30]
18. Install **MATLAB** from https://ww2.mathworks.cn/products/matlab.html.

Note: A MATLAB version newer than 2020a is necessary to enable the graphic user interface (GUI) of SMT_unwrapping.

19. Download SMT_unwrapping.zip from the Xiao Lab GitHub repository (https://doi.org/10.5281/zenodo.4306645). Unzip the file into a directory of your preference, and then add the SMT_unwrapping folder along with its subfolders to the default path of MATLAB by clicking "Add with subfolders" under the "Set Path" panel on the MATLAB homepage.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
One Shot PIR2 chemically competent *E. coli*	Thermo Fisher Scientific	C111110
BW25113 (*E. coli* K12 WT background)	GSGC	7636
EC1908 (*E. coli* MG1655 cells with FtsN depleted)	David Weiss Lab	Tarry et al.[31]
EC4440 (EC1908, pAH123)	David Weiss Lab	Lyu et al.[2]
Recombinant DNA		
pJB007 (TagRFP-T-FtsI, $lacI^{Q}$)	Jie Xiao Lab	Addgene 98995[15]
pXY349 (FtsW-TagRFP-T, $lacI^{Q\ mut}$)	Jie Xiao Lab	Addgene 200262[1]
pAH123	David Weiss Lab	Haldimann et al.[24]
pDSW2083 (Halo-$ftsN^{sw}$)	David Weiss Lab	Lyu et al.[2]
Chemicals, peptides, and recombinant proteins		
Janelia Fluor JF646 HaloTag ligand	Promega	GA1120
Janelia Fluor JFX650 HaloTag ligand	Promega	CS315104
SeaPlaque GTG Agarose	Lonza	50111
Alconox powdered precision cleaner	Alconox	1104-1
Potassium hydroxide	Merck	8143531000
Ethanol 99%+	Fisher scientific	10048291
M9, minimal salts, 5×	Merck	M6030
MEM amino acids (50×) solution	Merck	M5550
Calcium chloride solution	Merck	21115
Magnesium sulfate solution	Merck	M3409

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
D(+)-glucose anhydrous for molecular biology	BioFroxx	1179GR500
MOPS EZ Rich Defined Medium Kit	Teknova	M2105
Critical commercial assays		
In-Fusion HD Cloning Kit	Takara	639650
GeneJET Plasmid Miniprep Kit	Thermo Fisher Scientific	K0503
Software and algorithms		
Fiji or ImageJ	Fiji Team	https://fiji.sc/
MATLAB	MathWorks	https://www.mathworks.com/
MATLAB script package	This paper	https://doi.org/10.5281/zenodo.4306645
Other		
Stericup quick release-GP sterile vacuum filtration system	Merck	S2GPU05RE
Inoculating loop (sterile)	Merck	I8263
FCS2 chamber	Bioptechs	060319-2-03
TetraSpeck fluorescent microspheres with average diameter 0.1 μm	Thermo Fisher Scientific	T7279
40 mm round cover glass	Bioptechs	40-1313-03193
Adjustable cover-glass rack	Electron Microscopy Sciences	72243
Home-built microscope	Jie Xiao Lab	Table 1

MATERIALS AND EQUIPMENT

M9 minimal medium (100 mL)		
Reagent	Amount to add (for 100 mL)	Final concentration
M9 Salts (5×)	20 mL	1×
Glucose (20%)[a]	2 mL	0.4%
MEM Amino Acids (50×)	2 mL	1×
$MgSO_4$ (1 M)	200 μL	2 mM
$CaCl_2$ (1 M)	10 μL	100 μM
ddH_2O	75.8 mL	

[a]Filter-sterilize and store at 4°C.

EZ Rich Defined Medium (100 mL)		
Reagent	Amount to add (for 100 mL)	Final concentration
MOPS Modified Rich Buffer (10×)[a]	10 mL	1×
0.132 M Potassium Phosphate Dibasic Solution[a]	1 mL	1.32 mM
ACGU Solution (10×)	10 mL	1×
Supplement EZ (5×)[a]	20 mL	1×
20% Glucose [b]	1 mL	0.2%
ddH_2O	58 mL	

[a]from MOPS EZ Rich Defined Medium Kit from Teknova.
[b]Filter-sterilize and store at 4°C.

STEP-BY-STEP METHOD DETAILS

Sample preparation

Timing: 1 day

The purpose of this step is to culture *E. coli* cells expressing the FP/HaloTag-fused POI to the log phase for subsequent SMT imaging.

1. Prepare cell cultures in the log phase in a suitable growth medium, such as M9 or EZRDM, from frozen stocks.
   a. Streak cells from a frozen stock onto fresh LB plates with the appropriate antibiotics. Incubate the plates at 30°C or 37°C for 15–24 h.
   b. Using inoculating loops or pipette tips, pick up several single colonies from the LB plates and inoculate them into separate tubes containing 3 mL of growth medium. Allow the cells to grow with shaking at 220 rpm at 25°C for ~15 h (in M9) or ~5 h (in EZRDM).

   Note: Ensure the plate with single colonies is freshly prepared within one week.

   Note: The choice of growth medium and temperature can be adapted to suit the specific bacteria and experimental requirements. The growth time must be adjusted for bacterial division studies to maintain cells in the liquid culture during the early log phase (OD_{600} = 0.1–0.3).

   Note: It is recommended to select multiple colonies to increase the likelihood of successful growth in the subsequent step.

   c. Measure the OD_{600} of cultures to check their growth stages. If the OD_{600} is between 0.1 and 0.3, the culture is ready for step 2 (POI-HaloTag) or step 4 (POI-FP). If the OD_{600} is higher than 0.3, re-inoculate a small portion of the culture into a fresh growth medium to OD_{600} ~0.05. Monitor the OD_{600} every 30 min or 1 h until it reaches 0.1 to 0.3.

   Note: The vector (pCH) used in this protocol (key resources table) and Yang et al.[1] does not require IPTG induction in M9 medium to achieve a sufficiently low single-molecule level expression. For other vectors or media, however, induction may be necessary (we used 10 μM IPTG in the EZRDM medium). Users should inspect the expression level using fluorescence images and determine the optimal induction condition accordingly.

2. HaloTag labeling (For HaloTag fusion proteins only):
   a. Aspirate 1 mL of cell culture from step 1c to a 1.5 mL EP tube and add 0.1–10 μL of 1 μM JF646 (or JFX650) Halo ligand to a final concentration of 0.1–10 nM. Vortex the culture gently for a few seconds to ensure thorough mixing.

   Note: The ligand concentration requires optimization according to the labeling result. The final labeling density should be approximately one or two molecules per cell for effective single-molecule tracking. Typically, we maintain the same labeling time while adjusting the ligand concentration to achieve the desired single-molecule labeling density.

   b. Cover the tube with alumina foil. Incubate the cells for 30 min at 25°C with gentle rocking.
   c. Wash the cells three times using 1 mL or 500 μL of the imaging medium or buffer (e.g., M9). Incubate the cells at 25°C for 5 min with gentle rocking during each wash. Pellet the cells with a bench-top spinner or centrifuge at ~8000 g for 2 min.

   Note: The washing step could be further optimized depending on the specific bacterial species used. For instance, we have found that the 5-min incubation is unnecessary for labeling FtsW-HaloTag in *Caulobacter crescentus* cells.[3] Readers can assess the efficiency of the washing step by imaging WT cells following the same treatment. WT cells lacking the HaloTag fusion proteins should not exhibit observable fluorescence signals.

   d. Resuspend the cell pellet from the final wash in ~50 μL fresh culture medium (M9 or EZRDM) or other fresh media used for cell growth for imaging. Measure and adjust the OD_{600} of the final culture to fall within the range of 1–3.
3. While preparing the cell culture, make a gel pad for imaging (Methods video S1, related to steps a-d).

a. Gently mix ~10 mg of SeaPlaque GTG low melting temperature Agarose in ~500 μL of M9 or EZRDM to achieve a final concentration of 2% (W/V).
b. Heat the mixture at 70°C in a metal heat block or water bath for half an hour. The melted agarose gel can be stored at 50°C for up to 5 h.
c. Place the microaqueduct slide (from the FCS2 chamber) on the benchtop with a 0.75-mm thick gasket. Apply 50–100 μL of the melted agarose gel to the center of the slide. Gently cover the droplet of gel with a pre-cleaned cover glass immediately.
d. Allow the gel to solidify at 25°C for at least 30 min.

⚠ CRITICAL: To minimize potential drifting of the gel pad during SMT imaging, it is recommended to prepare the gel pad 2–5 h before imaging and store it in a humidified box (e.g., an empty tip box filled with water) near the microscope to avoid abrupt temperature fluctuations. Start imaging 20–30 min after the sample or the imaging chamber has been assembled and settled on the microscope.

4. Apply the cell culture from step 1c or 2d onto the gel pad prepared in step 3 (Methods video S1, related to steps a-d).
   a. Carefully peel the cover glass from the agarose gel pad using a razor blade.
   b. Immediately apply 0.5–1 μL of the sample onto the top of the gel pad.
   c. Wait 2–3 min, and cover it with a new, clean, dry glass.
   d. Assemble the FCS chamber according to the manufacturer's instructions (https://bioptechs.com/product/fcs2-chamber/).

Single-molecule imaging

Timing: 1–2 h

The purpose of this step is to detect single molecules of interest and track their movement in living bacteria cells.

5. Securely position the sample chamber on the microscope stage. Visualize cells in bright-field mode to ensure they are appropriately settled on the gel pad and display the expected morphology.

   Note: If cells exhibit abnormal morphologies, stop the single-molecule tracking experiment and specify the underlying causes. Only proceed to the imaging experiment when cell grow and divide in predicted phenotypes.

   Note: Make sure that cells do **NOT** move or drift on the gel pad! Accurate tracking of single molecules is not possible in moving cells. Take the time to search around the gel pad to find a field of view where cells remain still. If a suitable region cannot be located, consider preparing a new gel pad by carefully adjusting conditions in step 3–5, such as the size of the gel pad and the drying time for the topped cell culture. We have observed that a larger gel pad generally reduces cell movement. Adjust the time after applying cell culture based on the environment temperature and humidity; excessively wet or dry conditions may lead to cell movement or clumping on the gel pad.

6. Measure the laser power and calculate the power density.
   a. Switch the illumination mode to Epi, which allows the laser beam to pass through the sample perpendicularly.
   b. Set the laser output power to a range of 10–100 mW and measure the laser power at the top of the sample.

c. Capture a snapshot of the sample, identifying the illumination region as the portion of the image with a high background.
d. The power density is then calculated by dividing the laser power by the area of the illumination region.
e. Adjust the excitation power density by tuning the laser output or adding a neutral density filter (DNF1) in the excitation optical path (Figure 2).

Note: It is essential to measure and adjust the excitation power density to 1) achieve a good signal-to-noise ratio for single-molecule detection and localization and 2) avoid rapid photobleaching. Our previous studies used 1–2 W/cm^2 excitation power density for TagRFP-T or JF646 with 0.5–1 second exposure time. Increase the laser power if the molecules move faster, as a shorter exposure time is required. For example, an exposure time of 50 ms may require an excitation power density of 100–500 W/cm^2.

7. Take streaming SMT movies.
   a. Switch the illumination to HILO mode by laterally moving the stage until the incident angle of the laser beam reaches 60°–70°.

   Note: TIRF illumination mode is not utilized in this application because proteins within highly constricted septa cannot be observed with the shallow excitation field of TIRFs.

   b. Focus at ~200 nm above the bottom of cells (about a quarter of an *E. coli* cell or half of a *C. crescentus* cell).
   c. Capture images continuously with an exposure time of 0.5–1 s for 3–5 min.
   d. Take bright-field images before and after fluorescence imaging and compare the positions of the cells from the bright-field images. Cells with apparent movement or drifting are excluded from further data processing steps.
   e. For 3D-SMT, Z-position calibration is performed by imaging 1:500 diluted TetraSpeck fluorescent microspheres with an average diameter of 0.1 μm. Capture 100 Z-stacks around the spheres with a step size of 20 nm[8].

   Note: The width of *E. coli* cells (~1 μm) exceeds the depth of field of most high NA objectives (~600–700 nm). The top and bottom of the cell will be blurry if the focus is set in the middle of the cells. To obtain more trajectories moving in the lateral X-Y plane, which provides a higher resolution than the vertical Z-direction, we focus near the cell's bottom (or the top). For small bacteria such as *C. crescentus* (~500 nm cell width), we focus on the mid cell to monitor all molecules across the septum.[3]

   Note: A longer exposure time (or slower frame rate) helps track molecules with slow movement and filter out molecules diffusing rapidly along the cylindrical part of the cell body, such as FtsI outside the divisome (~0.04–0.1 μm^2/s).[16]

Single-molecule tracking analysis I: Localization

Timing: 2–4 h

The purpose of this step is to detect single-molecule signals in each frame and accurately determine the central position of each molecule. The resulting coordinates of molecules will be used in subsequent steps.

The first step of single-molecule analysis is to localize each molecule with a high spatial resolution in either 2D or 3D. The input of this step is the multi-frame .tif or .tiff files generated from the **single-molecule imaging** step (Figure 3).

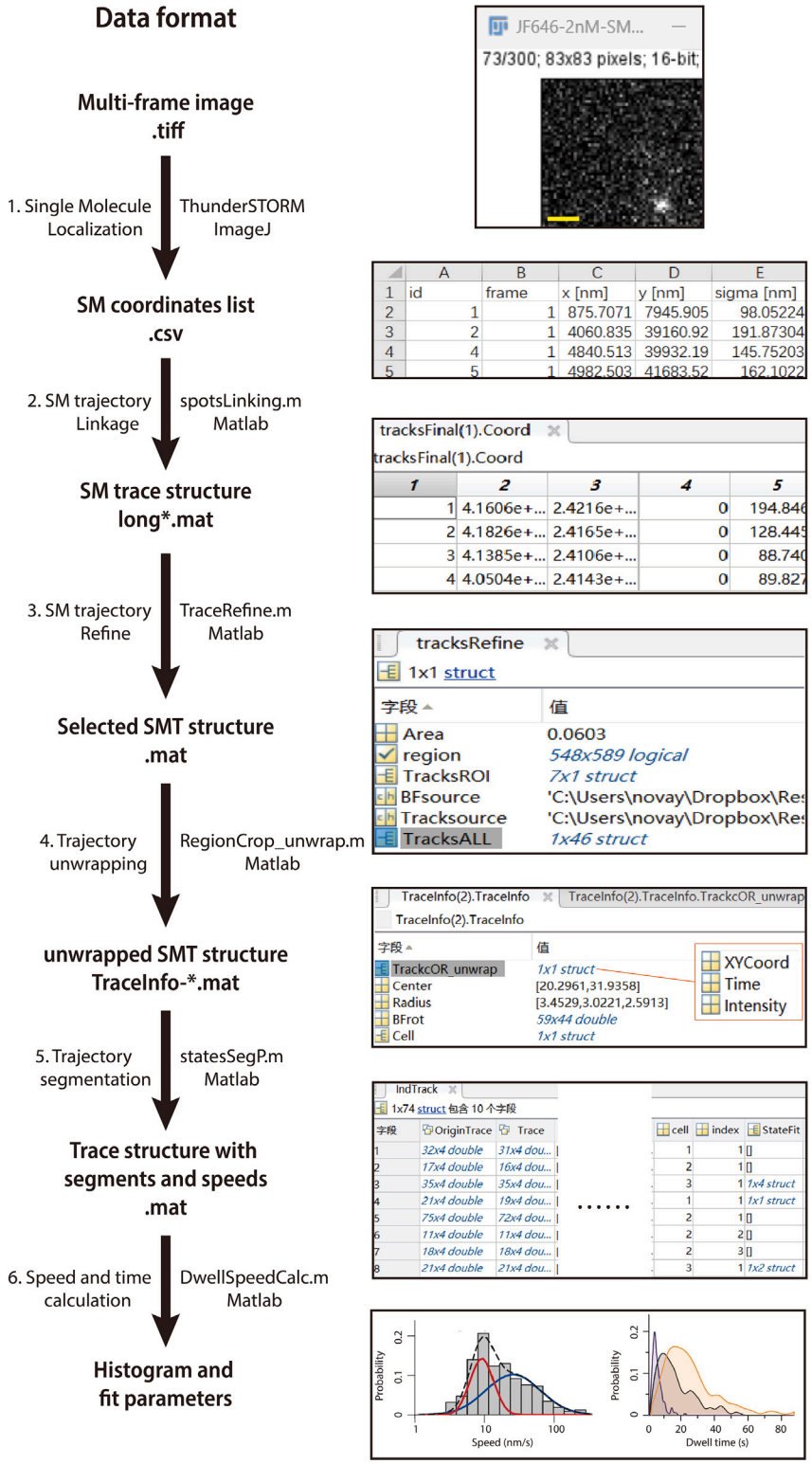

Figure 3. A step-by-step demonstration of the single-molecule tracking data analysis procedure

Steps 1 and 2 correspond to Single-molecule tracking analysis I-II, while steps 3 and 4 are discussed in section Single-molecule tracking analysis III. Steps 5 and 6 are described in Motion state classification and speed calculation I and II. The "App or Code" column showed the main code used in each step. The "Data format" column presents the input

Figure 3. *Continued*
and output data or files for every step. The representative outlooks are shown on the right. A detailed user video for the code (Step-by-step Single-Molecule Tracking analysis) can be found here: https://doi.org/10.5281/zenodo.4306645. Scale bar, 1 μm. Reprinted with permission from Yang et al., Nature Microbiology (Copyright 2021).[1] Permission to reuse this figure has been obtained from Springer Nature.

8. Follow the video (Methods video S2) to customize the ImageJ macro file (FtsW-RFP-singleMoleculeLoc-Macro.txt) to run the ThunderSTORM plugin. It is necessary to specify camera setup parameters (such as the EM gain, pixel size, etc.) and localization parameters (such as intensity threshold and fitting method).

   Note: Detailed descriptions of all the parameters can be found in the help file of the tutorials of the ThunderSTORM plugin.

   Note: Enabling the multi-emitter detection algorithm is unnecessary, given the relatively low molecule density.

   Note: The maximum likelihood estimator can enhance localization accuracy but significantly reduce computing speed.

   Note: We use the ThunderSTORM plugin developed in the Hagen lab in this protocol. Alternative algorithms or software (such as SMAP[32]) can also generate spatial coordinates of single molecules, especially when the images are captured by sCMOS cameras. These images exhibit pixel-dependent noise, unlike EMCCDs, which have a uniform noise distribution.[33] The user, however, would need to convert the output list of molecular coordinates into a format similar to ThunderSTORM (Figure 3, a .csv file with head labels such as '*id*,' '*frame*,' etc.).

9. Prepare directories on the computer for batch-processing using ThunderSTORM.
   a. Create a separate folder to store all the single-molecule images (.tif files) from experiments conducted under the same conditions.
   b. Create another folder to save the resulting localization lists.
10. For 3D-SMT data, generate the calibration curve using the Z-stack images of the TetraSpeck fluorescent microspheres from step 7e, following the tutorials of the ThunderSTORM plugin.
11. In the ImageJ menu, click *Plugin>Macros>Install*. Select the modified macro file.
12. Click the button with the name of the macro file appearing at the bottom of the *Plugin>Macros*.
    a. Select the folder with .tif files (step 9).
    b. Select the second folder to save the results, which will be saved as .csv files.

    Note: Depending on the data size and computer speed, the process will initiate and may take several hours on a personal computer.

    Pause point: Step 12 may take several hours, up to one night, to complete the analysis for large data sets.

Single-molecule tracking analysis II: Tracking

Timing: 1–2 hours

The purpose of this step is to link localizations from the same molecules to construct single-molecule trajectories.

Some frames may contain more than one molecule; hence, the coordinates of the same molecule moving in different frames must be identified and linked to generate single-molecule trajectories. To achieve this goal, we implement the nearest-neighbor-finding algorithm.[34] After the linkage

step, we systematically filter out mis-linked trajectories that do not meet specific criteria, such as not residing within a cell, exhibiting dramatic intensity fluctuations between frames, or failing to occupy anticipated positions (e.g., the septum for divisome proteins). The process of this section is demonstrated in Methods video S2, related to steps 13–26.

13. Launch MATLAB and change the current directory to the folder containing all the result .csv files from step 12.
14. Run ***spotsLinking.m***.
15. Set the **Spatial Threshold** to ~300 nm, slightly exceeding the size of the microscope point spread function (PSF).

    Note: The **Spatial Threshold** value represents a molecule's maximum allowable distance between consecutive frames. Any spot appearing above this threshold will be treated as a different molecule. Molecules moving too fast will be blurred out and cannot be detected in the **Single-molecule tracking analysis I: Localization step** due to the long exposure time.

16. Set the **Time Threshold** to a large value (i.e., 30 or 50 frames). \

    Note: This threshold represents the maximum permissible dark interval (in frame numbers) for linking two molecules, provided they are also sufficiently close in space (i.e., < **Spatial Threshold**). Initially, set a high value to characterize the off-time distribution under the current imaging condition. This threshold value will be adjusted in step 19.

17. Set the **Weight of Z** to 0 (for 2D imaging) or 1 (for 3D imaging) according to the experimental setup.
18. Click **Load Coordinate files** and select all the .csv files for analysis.

    Note: This step may take a few minutes and generate a distribution of the off-time (dark frame number) between two localizations in proximity. The average off-time will be displayed as the "Mean Lifetime" at the top of the window.

19. Based on the calculated average off-time, readjust the **Time Threshold** to a reasonable value (2–3 times the average off-time is suggested, e.g., five frames for our FtsW-TagRFP-T tracking and eight frames for our HaloTag-JF646-FtsN tracking).

    Note: Other reasonable thresholds can be used. Maintain consistency across all files of the same experiment.

20. Set the **Minimal Trace length** to an appropriate value, which determines the shortest trajectory length.

    Note: In our work, we employed a 10-frame threshold, twice the length of our shortest segment (5 frames).

    Note: The code saves all trajectories and those exceeding the designated thresholds in two separate sets of files. You may choose either one for subsequent data processing.

21. Click **Link the spots and Save file.** The GUI will use the current parameters on the panel to process all loaded files and save linked trajectories from each file in a new file.

    Note: The localizations will be saved in .mat format named "Coord-*.mat". Linked trajectories (those longer than the **Minimal Trace length**) will be saved in .mat files called as "Long-*.mat".

    Optional: If you possess data in multiple folders, you can process each folder individually. Afterwards, click "Combine files" to merge those data structures into one. Alternatively, you can

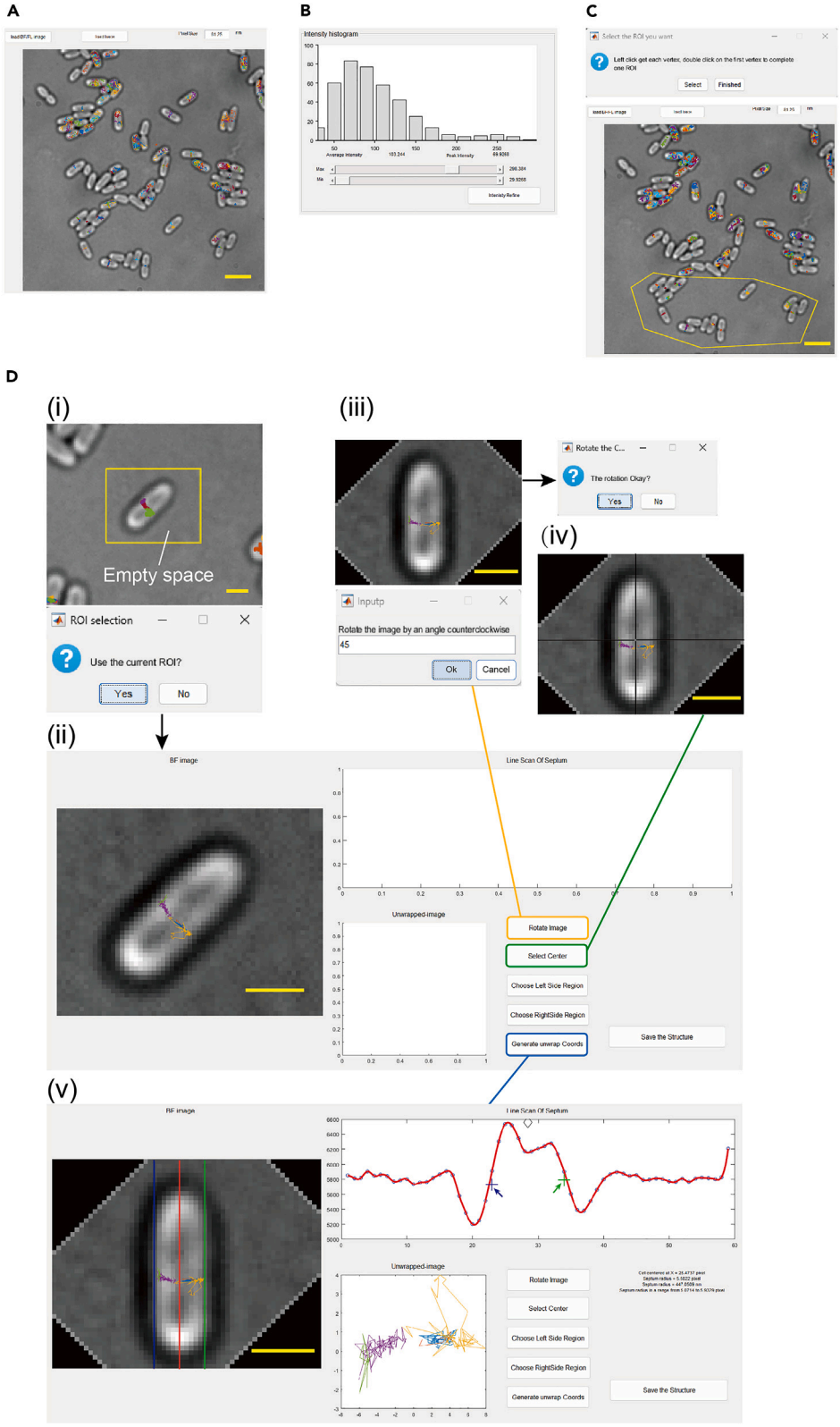
A
B
Intensity histogram
C
Select the ROI you want
Left click get each vertex, double click on the first vertex to complete one ROI
Select
Finished
D
(i)
Empty space
ROI selection
Use the current ROI?
Yes
No
(ii)
BF image
Line Scan Of Septum
Unwrapped-image
Rotate Image
Select Center
Choose Left Side Region
Choose RightSide Region
Generate unwrap Coords
Save the Structure
(iii)
Rotate the C...
The rotation Okay?
Yes
No
Inputp
Rotate the image by an angle counterclockwise
45
Ok
Cancel
(iv)
(v)
BF image
Line Scan Of Septum
Unwrapped-image
Rotate Image
Select Center
Choose Left Side Region
Choose RightSide Region
Generate unwrap Coords
Save the Structure

Figure 4. Demonstration of the SMT data analysis GUI

(A–C) The GUI of ***TraceRefine.m***. (A) Color-coded traces superimposed on the bright-field image. Scale bar, 3 μm. (B) A histogram of the average intensity of each trajectory, which can be adjusted to filter out overly bright or dim molecules. (C) A hand-drawn polygon is used to enclose trajectories within ROI. Scale bar, 3 μm.

(D) Screenshot from ***RegionCrop_unwrap.m***: (i) A cropped rectangular ROI containing an entire cell in the center, with sufficient space around it. (ii) The selected cell and its associated trajectories are displayed in a new window. (iii) The "**Rotate Image**" option allows users to specify an angle in degrees (positive values indicate a counterclockwise rotation) to align the cell's long axis vertically. (iv) The "**Select Center**" function enables users to click and point out the center of the septum. (v) The "**Generate Unwrap Coords**" option allows users to define the width of the cell (or the septum). Users click on the cross points of the red scanline and the left plateau first (indicated by blue arrows), followed by the cross point on the right side (indicated by green arrows). The GUI marks these selected cross points on the line and the bright-field image using blue and green lines. Scale bars, 1 μm.

perform this combination at the end of the **Single-molecule tracking analysis III: Cell envelope unwrapping** section.

22. Run ***TraceRefine.m*** in MATLAB. A new GUI will appear to assist in the interactive removal of "bad trajectories". Change the **Pixel Size** according to the microscope setting for precise bright-field image and trajectories overlay.
23. Click **Load BF/FL image** and **Load trace** to load the bright-field image and its corresponding trajectory file from step 19 ("Long-*.mat").

Note: Single-molecule traces are color-coded in time and superimposed on the bright-field image (Figure 4A, Methods video S2). The histogram of the average intensity of each trajectory is automatically calculated and displayed on the right panel.

24. To filter out trajectories with high intensity (likely from aggregates) or low intensity (likely from noise), drag the **Max** and **Min** bar to adjust the intensity thresholds. Then click **Intensity Refine** to remove all too bright or dim molecules (Figure 4B, Methods video S2).
25. Click **Select ROI.** The GUI will enable you to draw polygons that enclose trajectories in the regions of interest (ROI) (Figure 4C, Methods video S2).
26. Click **Save Selected Traces** to save the chosen trajectories. Optional: click **Save image** to save the current GUI interface.

Note: "Bad trajectories" refer to trajectories of background objects (such as dust particles outside cells), molecules not in the septum region of the cell (for divisome proteins), or multi-molecule aggregations (displaying high intensity).

Pause point: Steps 22–26 should be applied to all result files. You may pause at step 26 as long as the trajectories are saved.

Single-molecule tracking analysis III: Cell envelope unwrapping

Timing: 1–4 h

The purpose of this step is to flatten the trajectories along the curved cylindrical surface of a cell.

In typical imaging setups (except for the micropillar setup[9,16,35]), bacterial cells lie flat on the cover glass. As single molecules traverse the cell membrane, which is a curved surface, the motion of divisome proteins like FtsW and FtsI may be underestimated. To address this curvature effect, we calibrated the diameter of the cell (or the septal ring) using the bright-field image.[1] In 2D tracking, the "real trajectories" can be back-calculated geometrically using the position of the cell center and its diameter. In this step, we also decompose (or project) the trajectories onto two orthogonal axes: the long and short axes of the cell. In 3D-SMT, we fit the trajectories onto a circle determined by the cell center and diameter as those in the 2D scenario. We have observed comparable SMT results for membrane proteins in both 2D and 3D modes. However, if

the protein is not localized on the cell envelope and does not exhibit membrane-bound movement, 3D-tracking should be the preferred technique.

For 2D-tracking *(steps 27–31)*

27. Run ***RegionCrop_unwrap.m*** to initiate a new GUI. Adjust the **PixelSize** if necessary. Enter the date of the experiment or data processing to replace the YYYYMMDD at the top of the GUI. Then click **Load BF** and **Load Traj** to input the bright-field image and its trajectory file from step 26.

    Note: The trajectories will be superimposed on the bright-field image, each displayed in a distinct color.

28. Click **Select ROI.** The GUI will prompt you to crop a rectangular ROI. Click on a specific cell's top-left and bottom-right corners, ensuring it encompasses an entire cell in the center with surrounding empty spaces of approximately 1–2 cells (Figure 4D (i), Methods video S2).

    Note: The chosen cell and its associated trajectories will be displayed in a new window (Figure 4D (ii), Methods video S2).

29. Click **Rotate Image** and input an angle in degrees (positive numbers mean counter-clockwise rotation) to align the cell's long axis vertically (Figure 4D (iii), Methods video S2).
30. Click **Select Center** and then click on the center of the septum on the bright-field image as accurately as possible (Figure 4D (iv), Methods video S2).

    Note: A smoothed profile of the bright-field image along the short axis through the center will appear in the top-right panel (in red).

    Optional: **Choose Left Side Region** and **Choose Right Side Region** are tools for calculating the bright-field image's left- and right-side plateau. They are used for visualization in the subsequent step.

31. Unwrap and save the coordinates:
    a. Click Generate Unwrap Coords.
    b. Click on the cross points of the red scanline and the left plateau first, followed by the cross point on the right side. The GUI will mark the selected cross points on the line and also on the bright-field image (in blue and green lines) (Figure 4D (v), Methods video S2).
    c. Repeat this step if the selection appears unsatisfactory.
    d. Click **Save the Structure**, and the data will be saved, returning the GUI to its main interface.

For 3D-tracking *(step 32–39):*

32. Run ***RegionCrop_unwrap3D.m*** to initiate a new GUI.
    a. Adjust the **PixelSize** if necessary.
    b. Enter the date of the experiment or data processing to replace the YYYYMMDD at the top of the GUI.
    c. Click **Load BF** and **Load Traj** to input the bright-field image and its filtered trajectory file from step 24. The trajectories will be superimposed on the bright-field image, each displayed in a distinct color (Figure 4A) (similar to step 25).
33. Click **Select ROI.** The GUI will prompt you to crop a rectangular ROI. Then click on a specific cell's top-left and bottom-right corners, ensuring it encompasses an entire cell in the center with surrounding empty spaces of approximately 1–2 cells (Figure 4D (i)).

    Note: The chosen cell and its associated trajectories will be displayed in a new window (Figure 4D (ii)).

34. Click **Rotate Image** and input in an angle in degrees (positive numbers mean counter-clockwise rotation) to align the cell's long axis vertically (Figure 4D (iii)).
35. Click **Select Center of Ring** and then click on the center of the septum on the bright-field image as accurately as possible (Figure 4D (iv)).

Note: A smoothed profile of the bright-field image along the short axis through the center will appear at the top-right panel (in red).

Optional: **Choose Left Side Region** and **Choose Right Side Region** are tools for calculating the bright-field image's left- and right-side plateau. They are used for visualization in the subsequent step.

36. Click **Generate Ring Diameter.** Click on the cross points of the red scanline and the left plateau first, followed by the cross point on the right side. The GUI will mark the selected cross points on the line and also on the bright-field image (in blue and green lines) (Figure 4D (v)). Repeat this step if the selection appears unsatisfactory.
37. Click **Select Center of Cell** and then click on the center of the non-constricted cell cylinder on the bright-field image as accurately as possible. A smoothed profile of the bright-field image along the short axis through the center will appear at the top-right panel (in red).

Optional: **Choose Left Side Region** and **Choose Right Side Region** are tools for calculating the bright-field image's left- and right-side plateau. They are used for visualization in the subsequent step.

38. Click **Generate Cell Diameter.** Click on the cross points of the red scanline and the left plateau first, followed by the cross point on the right side. The GUI will mark the selected cross points on the line and the bright-field image (in yellow and black lines). Repeat this step if the selection appears unsatisfactory.
39. Click **Save the Structure**, and the data will be saved, returning the GUI to its main interface.

Note: We estimate the cell width (diameter) from the cross points with a correction coefficient measured from PALM images of the Z-ring in our lab.[8] It might introduce some bias when using different illumination modes, such as phase contrast. However, the unwrapping procedure to correct trajectories generally produces results closer to the "truth" than the raw data.

40. Repeat steps 27–31 for 2D-tracking or steps 32–39 for 3D-tracking until all cells with trajectories are processed.

Note: To redo the unwrapping step for a specific cell, left-click the item in the region list on the right panel and click **Delete trace** to remove it.

41. Click **Save Data** to auto-save all the rotated trajectories with the cropped bright-field images in the data structure. Click **Delete all** to clear the workspace before processing the next file. The data structure should be saved as "YYYYMMDD-TraceInfo-*.mat".

Pause point: This step completes single-molecule trajectory processing. The next section is specific for trajectory segmentation, classification, and directional speed calculation of divisome proteins.

Motion state classification and speed calculation I: Trajectory segmentation

Timing: 1–3 days

The purpose of this step is to segment the trajectories based on their motion properties.

When using a long exposure time (0.5–1 s), most freely diffusing molecules become blurred and cannot be localized. We have developed an analysis pipeline to segment the trajectories and differentiate between stationary and progressively moving molecules. Initially, an unwrapped trajectory along the septum is plotted against time. Users can visually inspect the shape of the trajectory and select the most distinct segment with a unique motion state. The algorithm then calculates the probability of this segment representing directional movement based on experimental and simulated displacements of various moving states (see details in the Methods part and Figure S3 in Yang et al.).[1] Users can then determine whether the segmentation is reasonable (proceed to the next segment) or not (redo the segmentation). This section can be performed iteratively to approach optimal segmentations.

42. Generate a simulated set of stationary trajectories with varying lengths using **Section 0** of the ***statesSegP.m*** file.

    Note: The results of this section will be saved as "SimuFtsW.mat". The variable *filenameSimu* in line 21 can be customized according to individual experiments. These simulated trajectories will be used in step 46 to calculate the likelihood of a segment being stationary (confined diffusion) or directional motion (Methods video S2).

    Note: Given a segment of a trajectory, we fit its position against time using a linear function and calculate the overall displacement. To distinguish segments indicating directional movement from those of stationary state (confined diffusion), we primarily assess whether the displacement exceeds random fluctuations attributable to diffusion and localization uncertainty. As these fluctuations are stochastic in nature, we can only calculate the distribution of these fluctuations and estimate the probability of a segment to be stationary or directional moving. Therefore, we initiate a simulation involving a set of confined diffusion segments to establish the displacement distribution for these stationary molecules. More details of the statistical test are described in the supplementary discussion in Yang et al.[1]

    Note: We fix the diffusion coefficient and confinement size to simplify the simulation. The parameters in the MATLAB code have been optimized for our study of cell division proteins FtsW, FtsI, and FtsN in *E. coli*. Specifically, we used the diffusion coefficient (*D*) of 0.0005 $\mu m^2/s$, based on FtsZ's *D*,[12] a confinement size (*B*) of 200 nm, reflecting the size of FtsZ clusters (200 nm[7]), and a localization error (*L_err*) of 15 nm in consideration of the single-molecule localization precision. The frame-to-frame time interval, ExpT, should be adjusted according to the specific experimental setup (measured in seconds).

    Note: Users can modify these parameters according to their unique biological systems and experimental conditions. We recommend initiating with stringent requirements, such as a low diffusion coefficient and short confinement length, for an initial simulation (or utilizing our current settings). Subsequently, employ these simulated trajectories to perform segmentation in steps 43–46 on a small portion of the data to identify all "stationary" segments. These initial segments can then be used to determine the actual *D*, *B*, and *L_err* values from real data, as outlined in step 41. Subsequently, rerun the simulation using the experimental *D* and *L_err* values. We have found this approach to be highly effective and robust for segmentation.

    ⏸ **Pause point:** The simulation can be time-consuming, potentially taking over 10 h on a personal laptop. A small sample size (*N_traj* = 100 or 200) can expedite the computation.

43. Interactively configure additional parameters for trajectory segmentation based on experimental data.
    a. In **Section 1** of ***statesSegP.m***, edit the "*Experiment*" variable to "2D_Tracking" or "3D_Tracking" according to the experiment.
    b. Adjust "*ExpT*" (time interval in seconds) and "*PixelS*" (pixel size, in nm) if necessary.

c. Run **Section 1** and select all trajectory files from step 41 (of the same experimental condition). A window will appear, allowing users to examine the distribution of the fluorescence intensity of individual localizations.
d. Click anywhere in this figure and input a number, *A*. All localizations with intensity greater than $Mo_I + A \cdot \sigma_I$ will be removed. Mo_I is the statistical mode of the intensity and σ_I is the standard deviation of the intensity. In our FtsW tracking studies, *A* was set to be 2.[1]

Note: High-intensity localizations could indicate abnormal aggregation or multiple molecules in the same frame. The code removes these localizations while retaining other points in the trajectory.

e. The script will then calculate the MSD curves in the X-axis (short axis of the cell) and Y-axis (long axis of the cell) for all trajectories. Click anywhere on the MSD plot to continue. The code will prompt users to input two parameters. The **noise level of X** provides guidelines for **segmentations** in step 46. This number should be similar to or ~ 10%–20% larger than the first point of the MSD in the X-axis, aiding in determining if a molecule has moved too far from its previous position.

Note: FtsZ clusters confine molecules in the division site or septum. Large displacements suggest a transition to another state or location, indicating the trajectory segmentation at that time point.

Note: The **Confinement of Y** estimates the width of the septum. This parameter is critical as points outside the septum will be removed. Set this number to be similar to the plateau of the MSD in the Y-axis or the known Z-ring width, for example, 100 nm in WT *E. coli* cells.

f. Save the refined and combined data structure.

44. Run **Section 2** of ***statesSegP.m*** to prepare for segmentation. Confirm that *Input_Params.ExpT* and *Input_Params.PixelS* are correct according to the exposure time and pixel size used in the experiment. The *Input_Params.TimeRange* should cover the maximum length of the trajectories.
45. Select the experimental dataset from step 43 and the simulation dataset from step 42 in the pop-up window.

Note: A new variable called ***IndTrack***, which contains all trajectory information, should be generated in the workspace. The length of ***IndTrack*** corresponds to the number of trajectories.

46. Change the ***Index*** from 1 to the length of ***IndTrack*** and run **Section 3** of ***statesSegP.m***.

Note: For each trajectory (Index), a GUI will appear, displaying the bright-field image of the cell with a color-coded trajectory (from blue to red, indicating the beginning to the end) in the top-left corner. Users can segment the trajectory state-by-state following the instructions provided in the pop-up box (Methods video S2):

a. Select one part of the trajectory located on the septum (or a position of interest) by clicking **Okay** in the pop-up box and then clicking on the two ends of the region you want to choose. If the entire trajectory is correctly localized in the septal region, click ***Useall***, and the code will use all the positions for calculation. If the trajectory is not in the area of interest (out of the septum), click ***Bad Trajectory*** to exit and continue analyzing the next one.

Note: The trajectory along the short axis will be displayed in the upper right region. Examine the trajectory by eye and approximately pinpoint the intervals characterized by a consistent speed, or uniform slope.

b. Click on the tentative start and end positions of one interval. Click "No" in the pop-up window if the segmentation looks unreasonable and redo this step.

Note: The linear fit will be displayed on top of the trajectory. The code will automatically calculate the probability distribution of *R* by bootstrapping and display it with that of the simulated stationary segment from step 42 on the bottom left region. Users can inspect the speed (*V*), *R-value*, and the probability of the segment being directional (*P_processive*). For more details on these parameters, refer to Yang et al.[1]

c. Click "Yes" to proceed to identify the next possible segmentation with a uniform slope. Repeat steps a-c until the entire trajectory is segmented.

⚠ CRITICAL: We strongly recommend declassifying trajectories from different experiments before proceeding to step 46. By doing so, the user will not know which experiment or condition the trajectories originate from. For instance, one user could create separate folders to store the IndTrack structures, while another can conduct the segmentation and identification process. This strategy mimics a double-blind test, effectively mitigating potential human bias.

Pause point: Step 46 may take a long time (ranging from hours to days) depending on the size of the dataset. The code automatically saves the ***IndTrack*** variable to the same file each time you run **Section 3**. Therefore, you can stop at any index of the trajectories and resume the analysis by reloading the ***IndTrack*** file.

Motion state classification and speed calculation II: Post-processing

Timing: 1–4 hours

The purpose of this step is to analyze the speed, lifetime, and percentage of different motion states.

To further reduce potential human bias, we apply the same criteria to re-classify all segments of the same protein fusion from different experimental conditions. For instance, our FtsW tracking experiment maintains consistency in threshold values for *R* and *P_processive* across all conditions, including WT or FtsZ mutant strains, drug treatments, and varying growth conditions.

47. Open the ***DwellSpeedCalc.m*** file; adjust the segmentation parameters; then run the code.

Note: **Rmax1** and **Pmin** are key parameters: any segment with *R* smaller than **Rmax1** and *P_processive* greater than **Pmin** will be classified as directionally moving. Some directionally moving segments with a long lifetime but slow speed may generate a small *P_processive*. Therefore, we also classify all segments with *R* smaller than **Rmax2** as directionally moving. Set the number of bins (**Nbin**) and the region of speed (**LowB** to **HighB**) for speed distribution plotting. We recommend using predefined parameters, specifically **Pmin** = 0.75, **Rmax1** = 0.5, and **Rmax2** = 0.2. These parameters can be adjusted in cases of noticeable mis-segmentation. A higher **Pmin** can reject more segments from the moving state. It is crucial to maintain parameter consistency across all experimental conditions.

48. Select segments classified from step 46 (the user can choose multiple files from the same condition). Save the variables containing the apparent moving speed and lifetime of each segment.

Note: The segments are now classified as directional and non-directional. The speeds of directional segments are saved in ***Vx***. The lifetimes are saved in ***Dtx*** and ***Dtf,*** respectively.

Note: The classification result (**R** and **P** scattered plot), speed distribution (in logarithm scale), and dwell time distribution (for both stationary and moving states) will be plotted and saved.

49. Perform non-linear fitting of the speed distribution. Different division proteins can move in single- (FtsN) or multiple- (FtsW/I) modes.
    a. We fit the cumulative probability density function (CDF) of the speed to a single or double log-normal distribution in our work using **cdfFitting*2pop*.m.** This MATLAB script uses the ***Vx*** variable from step 48 with a customized bin number and speed region, which could be the same or different from step 47.
    b. In **Section 2** of this code, the CDF of the directional speed is calculated and fit to single- or double log-normal functions.
    c. The standard errors of the fitting parameters are estimated by bootstrapping in **Section 3.** (Additionally, users can also implement the Jacobian of fitting to estimate fitting errors using functions **log1CDFfit** and **log2CDFfit_free**).
    d. The histogram in the log scale of the experimental data and fitting curves are generated in **Section 4.** All the results are combined to save in a variable named **Result** (Table 2).

Note: The log-normal distribution is chosen empirically as it describes the experimentally observed distribution well. Users can use other models and their application/code to analyze the speed dataset. The single- and double-log-normal functions we use to fit the data are:

$$\mathrm{CDF}_{\mathrm{single}} = \mathrm{A}\cdot\left[1 + \mathrm{erf}\left(-\frac{\ln x - \mu}{\sigma\sqrt{2}}\right)\right]$$

and,

$$\mathrm{CDF}_{\mathrm{double}} = P_1\cdot\left[1 + \mathrm{erf}\left(-\frac{\ln x - \mu_1}{\sigma_1\sqrt{2}}\right)\right] + (1 - P_1)\cdot\left[1 + \mathrm{erf}\left(-\frac{\ln x - \mu_2}{\sigma_2\sqrt{2}}\right)\right]$$

where erf is the Gauss error function. The median and mean of the speed can be estimated by $\exp(\mu)$ and $\exp\left(\mu + \frac{\sigma^2}{2}\right)$.

50. Calculate and fit MSD curves to obtain the diffusion and confinement information using ***MSDCalc.m.*** The corresponding results, including diffusion coefficient and confinement length, will be saved in variables listed in Table 2. Customized data analysis tools can be used to extract more information from these single-molecule trajectories.

EXPECTED OUTCOMES

First, a set of single-molecule trajectories of the POI are generated from step 39. These trajectories are decomposed along the septum and the long axis of the cell (Figures 2A and 2B in Yang et al.[1]). Second, processively moving and stationary segments from these trajectories are identified and classified into different states in subsequent steps. The speed distribution and multi-population fitting plot of the POI are similar to those shown in Figures 2d and 2, 3a–d in Yang et al.[1]). For FtsW, FtsI,[1,3] and FtsB,[14] we found that these proteins involved in sPG synthesis exhibit two states of movements: a fast state (~30 nm/s) driven by FtsZ's treadmilling and a slow state (~8 nm/s) coupled with the sPG synthesis reaction. However, their regulator, FtsN,[2] moves slowly at ~8 nm/s. Users will also obtain the distribution of the lifetime of the POI in stationary and moving modes, as shown in Figure 3E Yang et al.[1] The information about how long the POI stays in each state can help characterize the biochemical kinetics and mechanism by which the divisome is organized and dynamically regulated. Finally, users can perform additional quantification and statistical analyses to extract other information, such as transition kinetics from the single-molecule data, using other published or customized tools.

Table 2. Expected results from step 47–50

Name	Field	From	Description
Vx	–	DwellSpeedCalc.m	Speed of directional moving segments
Dtx	–		Dwell time of directional moving segments
Dtf	–		Dwell time of stationary segments
strucX	.TracksROI.Coordinates		Trajectories of directional moving segments [time, X, Y]
strucF	.TracksROI.Coordinates		Trajectories of stationary segments [time, X, Y]
Result	.CDF_real	cdfFitting2pop.m	Experimental CDF curve
	.CDF_real_fit1		Single population fitting curve
	.Residual1		Difference between single and experimental curve
	.CDF_real_fit2		Experimental CDF curve
	.P_fit1		Fitted parameter [A, μ, σ]
	.P_fit1_boot		Fitted parameters (1-pop) from bootstrapping
	.P_fit2		Fitted parameter [P_1, μ_1, σ_1, μ_2, σ_2]
	.P_fit2_boot		Fitted parameters (2-pop) from bootstrapping
	.Fit_Vdirx1		Mean speed and width from fitting [v_{mean}, v_{width}]
	.Fit_Vdirx1_sem		Standard error of Fit_Vdirx1
	.Fit_Vdirx1_boot		Bootstrapping results
	.Fit_Vdirx2		Mean speed and width from fitting [P_1, v_{mean1}, v_{width1}, v_{mean2}, v_{width2}]
	.Fit_Vdirx2_sem		Standard error of Fit_Vdirx2
	.Fit_Vdirx2_boot		Bootstrapping results
	.His_1pop_final		Histogram variable [x, $his_{experiment}$, his_{fit}]
	.His_2pop_final		Histogram variable [x, $his_{experiment}$, his_{fit_pop1}, his_{fit_pop2}, his_{fit_all}]
KusumiFit_x	.D_all	MSD_calc.m	MSD in x-direction [time (sec), MSD, MSD_{SEM}]
	.L		Confinement length of particle in the x-direction
	.sigma		Average displacement in x-direction
	.D		"Corrected" diffusion coefficient in x-direction
	.F		Kusumi fitting curve for the x-direction
KusumiFit_y	.D_all		MSD in y-direction [time (sec), MSD, MSD_{SEM}]
	.L		Confinement length of particle in the y-direction
	.sigma		Average displacement in y-direction
	.D		"Corrected" diffusion coefficient in y-direction
	.F		Kusumi fitting curve fit for the y-direction
s_xMSD	–		MSD in every interval time in x-direction [time, MSD, $MSD_{S.D.}$]
s_yMSD	–		MSD in every interval time in y-direction [time, MSD, $MSD_{S.D.}$]

LIMITATIONS

Our protocol has been implemented specifically to study divisome proteins in rod-shaped/like bacterial cells. However, in principle, we anticipate its application to other bacterial cells of different shapes (such as spiral or coccid). Users may need to justify the unwrapping step (steps 27–39) by employing super-resolution imaging of the Z-ring as described in the Methods of Yang et al.[1]

Up to this point, molecules utilized for segmentation and identification (step 46) are localized near the septum, exhibiting directional movement or slow diffusion in a confined area. Alternative methods should be employed to characterize their dynamic behaviors for freely diffusive molecules such as PBP1a and PBP1b in *E. coli*.[36–38]

TROUBLESHOOTING

Problem 1

The protein of interest POI may not be compatible with a HaloTag/FP fusion. TIn our experience, this is particularly true common for divisome proteins, since as many they are either membrane proteins are essential membrane or cytoskeletal proteins.

Potential solution

Tag POI at the opposite end (N- or C-terminus) using a different peptide linker may alleviate this problem. Alternatively, if there is a loop region distant from the essential and functional domains[39,40] of the protein, the tags could be inserted in that region, resulting in a sandwich fusion of POI.

Problem 2

Bacteria cells may move or drift during the time course of single-molecule imaging.

Potential solution

Two potential issues contribute to this problem. The first issue involves chamber and gel pad drift during the imaging period due to temperature instability. Since the temperature is often different between the wet lab and the microscope room, the hydrogel can expand or shrink after being assembled on the microscope, leading to cells drifting in the same direction with the gel pad in the field of view. To address this issue, we recommend incubating the gel pad in the microscope room, near the microscope, for a few hours to allow it to reach temperature equilibrium.

The second issue involves individual cells moving in different (often random) directions. This phenomenon is typically a result of an over-dried or overly wet gel pad. To mitigate this, try to adjust the time after applying the cell culture to the gel to reach a stable condition. The timing should be optimized based on the room's existing humidity and temperature conditions. We recommend conducting this step in the scope room, which usually maintains better temperature and humidity control than wet labs. If the sample is particularly valuable, consider searching the entire gel pad, as cells near the edge of the pad tend to be more stable.

Problem 3

High fluorescence background outside of the cells.

Potential solution

If there are fluorescent spots on the coverslip, it is advisable to optimize the cleaning procedure based on Xiao et al., 2008.[41] Store cleaned coverslips in ddH_2O and use ultra-pure N_2 to dry them before applying cells. Additionally, adjusting the microscope's focus to the top of the cell can help blur out background spots stuck on the cover glass. Another contributing factor could be the growth medium and imaging buffer. Complex media such as LB produce a high level of auto-fluorescence. Switching to chemically defined media such as M9 and EZRDM is recommended.

Problem 4

The density of fluorescent spots inside the cells is either too high or too low.

Potential solution

Optimize the expression level of the fusion protein containing HaloTag/FP by adjusting the inducer concentration. Researchers can also tune the expression level by modifying the promoter in their experiments.

When using the HaloTag as a reporter, titrating the dye concentration and incorporating additional washing steps (or extending the washing time) are recommended to reduce the number of spots in cells, especially for non-specific labels.

Problem 5

The ThunderSTORM plugin misses many single-molecule spots or identifies too many background low-intensity spots.

Potential solution

We recommend adjusting the threshold value in line 17 of the ImageJ macro file (originally named *FtsW-RFP-singleMoleculeLoc-Macro.txt*). Increase the threshold if you encounter false positive molecules, and decrease it if apparent single-molecule spots are not detected. However, it's important to keep the threshold within the range of 1–3.

If the threshold has to be set below 1, the background signal is often too high. Conversely, if the threshold must be set above 3, the expression or labeling level may be too high, which often results in the formation of bright aggregates.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact and corresponding author, Dr. Jie Xiao (xiao@jhmu.edu).

Technical contact

Further technical questions should be directed to technical contacts: Dr. Zhixin Lyu (jason.lyu@nih.gov) or Dr. Xinxing Yang (xinxingyang@ustc.edu.cn).

Materials availability

This study did not generate any new unique reagents.

Data and code availability

Scripts used in this study are available on Zenodo: https://doi.org/10.5281/zenodo.4306645.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102766.

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AUTHOR CONTRIBUTIONS

Conceptualization, J. Xiao, Z.L., and X.Y.; methodology, D.Y., Z.L., and X.Y.; software, D.Y., Z.L., and X.Y.; validation, D.Y., Z.L., and X.Y.; formal analysis, Z.L. and X.Y.; resources, D.Y., Z.L., and X.Y.; data curation, Z.L. and X.Y.; writing – original draft, X.Y.; writing – review and editing, D.Y., J. Xue, J. Xiao, Z.L., and X.Y.; visualization, D.Y., J. Xue, and Z.L.; supervision, J. Xiao and X.Y.; funding acquisition, J. Xiao and X.Y.

DECLARATION OF INTERESTS

The authors declare no competing interests.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work, the authors used ChatGPT 3.5 in order to improve the language. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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