DNA organization and segregation super-resolved

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Summary

With single-molecule localization microscopy (SMLM) it is possible to reveal the internal composition, architecture, and dynamics of molecular machines and large cellular complexes. SMLM remains technically challenging and frequently its implementation requires tailored experimental conditions that depend on the complexity of the subcellular structure of interest. Here, we describe two simple, robust and high-throughput protocols to study molecular motors and machineries responsible for chromosome transport and organization in bacteria using 2D- and 3D-SMLM.

Keywords: Photo-activated localization microscopy (PALM), *Bacillus subtilis*, Bacterial chromosome, Molecular motors, Sporulation, SpoIIIE, ParABS

1. Introduction

In vivo fluorescence microscopy enables the non-invasive observation of protein organization and localization in live cells with high specificity. However, the maximum resolution attainable in standard fluorescence microscopy is intrinsically limited by the diffraction of light. Considering the sizes of most machineries and subcellular structures, this limited spatial resolution (200–300 nm and 500–700 nm in the lateral and axial directions, respectively) hinders access to fundamental structural and mechanistic details. This limitation is considerably acute for bacteria, as the maximal resolutions are comparable to the size of the entire cell (typically ~1-2 μm). In recent years, the evolution of imaging hardware combined with the special properties of specific fluorescent probes, has allowed to detect individual molecules to overcome the diffraction barrier of light. Single-molecule localization microscopy (SMLM) methods combine the stochastic photo-activation of a single fluorophore per diffraction-limited volume at any given time and its localization with nanometer spatial resolutions. From the coordinates of all localized emitters, a reconstructed image at super-resolution (typically ~20-30 nm) can be obtained. Several SMLM strategies have been designed, and differ mostly in the fluorescent probe employed or in the mechanism for achieving stochastic photoactivation. Typical examples of SMLMs are Photo-activated localization microscopy (PALM/fPALM) and Stochastic Optical Reconstruction Microscopy (STORM/dSTORM) ***(1–5)***. Although the implementation of SMLM methods seems, at first, easily attainable, in practice its implementation is not straightforward. A truly multidisciplinary approach along with detailed and standardized protocols is required to guarantee high quality data acquisition and its proper interpretation.

The use of SMLM is starting to reveal details of the structure and dynamics of important bacterial machineries that were not previously accessible by conventional microscopy imaging methods ***(6–17)***. Here, using the bacterium *Bacillus subtilis* (*B. subtilis*) as a model system we present two protocols to perform SMLM imaging of molecular machineries involved in the folding and transport of DNA. First, we describe a microfluidics-based method to perform sequential multicolor imaging of bacterial chromosomes and membranes coupled to live PALM imaging of the membrane-anchored hexameric motor SpoIIIE, responsible of transporting directionally two-thirds of the *B. subtilis* chromosome into the nascent forespore before septum closing during sporulation ***(13)***. Secondly, we describe an alternative protocol to perform 3D PALM imaging in chemically fixed bacteria using agar pads as sample mounting devices. As a model structure, we use the kinetochore-like bacterial partition machinery constituted of a repeat of

DNA sequences (*parS*) to which a specific protein (ParB) binds to assemble a complex involved on the organization and segregation of the origin of replication region during bacterial vegetative growth and division ***(17)***. For both protocols, we give a detailed step-by-step guide, including cell culture, sample mounting, imaging conditions and final image reconstruction output. Finally, in the notes section we discuss several elements of the presented protocols that require special attention when employing super-resolution methodologies for bacteria.

2. Materials

Prepare all solutions using ultrapure water and analytical grade reagents when possible. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing of waste materials. Solutions percentage concentration are weight/volume (w/v %) unless otherwise indicated.

2.1. Bacterial strains

1. Control strain: wild type *B. subtilis* (PY79 background).
2. Strain carrying EosFP-labeled SpoIIIE: EB1407 (parental strain: PY79, genotype: *SpoIIIE-EosFP::Kan)* ***(13)***.
3. Strain carrying mEos2-labeled ParB: HM675 (parental strain: PY79, genotype: *spo0J-mEos2::neo*) ***(17)***.

2.2. Cell cultures

1. Luria-Bertani (LB) medium: 4 g NaCl, 4 g Tryptone, 2 g Yeast Extract, bring volume to 400 mL with water. For sporulating *B. subtilis* experiments, LB media is diluted 1/5 with sterilized water (LB 20%).
2. Sporulation solutions were adapted from ***(18)***. Solution A: 0.9 mL 2% $FeCl_3.6H_2O$, 0.83 g $MgCl_2.6H_2O$, 2.0 g $MnCl_2.4H_2O$, bring volume to 60 mL with water. Filter sterilize and store at 4°C (see Note 1). Solution B: 5.35 g NH4Cl, 1.06 g Na_2SO_4, 0.68 g KH_2PO_4, 0.97 g NH_4NO_3. Adjust pH to 7.0 with NaOH and bring volume to 100 mL with water. Aliquot and autoclave. Store at 4°C.
3. Pre-sporulation medium: Mix 50 ml of MOPS buffer 0.5 M pH 7.5 with 1 ml of Sporulation solution A and 10 mL of sporulation solution B. Bring volume to 1000 mL with water. Adjust pH to 7.4. Aliquot and filter in 92 mL fractions. Store at 4°C.
4. Sporulation medium: Mix 92 ml pre-sporulation medium with 2 ml 10% Glutamic Acid, 1 ml 0.1 M $CaCl_2$ and 4 ml 1 M $MgSO_4$.
5. Spizizen minimal medium (SMM): 0.2% $(NH_4)_2SO_4$, 1.4% KH_2PO_4, 0.6% K_2HPO_4, 0.1% sodium citrate dihydrate, 0.02% $MgSO_4$.
6. Synthetic media for *Bacillus subtilis* (SMBS): 0.05 mL 2 mg/mL Fe-NH4-citrate, 0.6 mL 1000 mM MgSO4, 0.1 mL 100 mM CaCl2, 0.2 mL 65 mM MnSO4, 0.1 mL 1000 µM ZnCl2, 0.1 mL Thiamine 2 mM, 2 mL 5% Glutamate, 7.5 mL 20%

Succinate. Bring volume to 100 mL with SMM solution. During the day carrying the experiment store at 4°C (see Notes 2-4).

7. Casamino Acids (CAA) supplement: 2 mg/ml. Store at 4°C.

2.2. Cells chemical fixation

1. 32% Paraformaldehyde (PFA).
2. 25% Glutaraldehyde.
3. 1M buffer phosphate pH 7.4.
4. Fixation Mix: 100 µl of 32% PFA, 0.8 µl of 25% Glutaraldehyde and 50 µl of water.

2.3. Agarose pads

1. Rectangular coverslip (#1.5H, Marienfeld).
2. 1 mm thick glass slide (SuperFrost, Ultra Plus, 25 x 75 mm)
3. Double-side adhesive tape (~1 mm thick).
4. Agarose (A4804, Sigma-Aldrich).
5. Spizizen minimal medium (SMM).

2.4. Microfluidic chambers

1. Rectangular coverslip (#1.5H, Marienfeld)
2. 1 mm thick glass slide (SuperFrost, Ultra Plus, 25 x 75 mm)
3. Parafilm M (P7793-1EA, Sigma-Aldrich)
4. Poly-L-Lysine (P4832, Sigma-Aldrich)
5. Chitosan (C3646-10G, Sigma-Aldrich)
6. Inlets (Intramedic PE Tubing, 0.011 in. (PE10) and PE Tubing, 0.023 in. (PE50)).
7. Syringes (1 mL BD tuberculin, 10 mL BD tuberculin).
8. Needles (BD PrecisionGlide Single-use Needles, 23G and 30G).

2.5. Microscopy components and setup

Figure 1A shows a simplified scheme depicting the microscope components and optical path (see Notes 5-6).

1. Lasers 405 nm and 641 nm (Vortran Technology, Stradus 405-100, Stradus 642-100).

2. Laser 488 nm (Coherent, Inc, Cube-488-50C).
3. Laser 561 nm (Sapphire 561LP, 150 mW, Coherent).
4. Laser 1064 nm (IPG Photonics Laser).
5. Objective OBJ (Nikon, Plan Apo VC 100x H).
6. Piezo stage PZ (Mad City Labs, Inc, Nano-drive 1).
7. Stage (Physick Instrumente PI, PI Mercury MS163E).
8. Camera C1 (Andor, iXon 897).
9. Camera C2 (Cooke, Pixelfly).
10. Dichroic mirrors DM1 (Semrock, 427 nm LaserMU, 503 nm LaserMU, 552 nm LaserMU).
11. Dichroic mirrors DM2 and DM3 (Chroma, zt/405/488/532/633rpcz1064rdc-sp).
12. Filter wheel FW (Thorlabs, ET605/70 nm, ET700/75 nm, FW102C).
13. Filters (Chroma, ET525/50 nm).
14. Data Acquisition DAQ (National Instruments, NI-USB 62-11).
15. Corrective MicAO 3D-SR system (Imagine Optic).
16. Homemade acquisition software controlling lasers, filter wheels and camera (LabView 2012, National Instruments).

2.6. Additional elements for sample preparation and software for data analysis

1. Fluorescent beads (40 nm, Trans FluoSpheres and 100 nm Tetraspeck, Invitrogen).
2. DNA labelling agent: SYTOX Green (S7020, Thermo Fisher Scientific).
3. Bacterial membrane dye: FM 4-64 (T-3166, Thermo Fisher Scientific).
4. CO_2 laser cutter (Thermoflan, France).
5. Bunsen burner.
6. Multiple Target Tracking (MTT) software for 2D single molecule detection ***(19)***.
7. Rapid STORM software for 3D single-molecule detection ***(20)***.
8. PALMCBS: home-made Matlab-coded software employed for data processing and image reconstruction ***(13, 21)***.

3. Methods

All steps are to be carried out at room temperature unless otherwise indicated.

3.1. Cell cultures

B. subtilis strains were stored in a mix of LB and glycerol (50%) at -80 °C. All cell culture steps should be carried under flame.

3.1.1. Sporulating B. subtilis

1. Streak EB1407 bacteria from -80°C glycerol stock onto solid (plate) Luria-Bertani (LB) medium (i.e. agar plate) complemented with 10 µg/ml kanamycin.
2. Culture agar plates 24 hours at 20°C.
3. Select a single colony from plate and perform at least 5 serial dilutions with LB 20% into sterile 50 ml falcon tubes (1/10 each in 5 ml final volume) and grow overnight at 30°C with agitation at 200 rpm (see Note 7).
4. The next morning measure the optical density (OD) at 600 nm and select dilution of bacteria exponentially growing (OD ~0.3-0.5), dilute to OD ~0.05 in LB 20% (25 ml final volume in 250 mL flasks for optimal aeration) and incubate at 37 °C with agitation at 200 rpm.
5. Measure OD at 1h intervals. When reaching OD ~0.6, centrifuge bacteria and remove supernatant (see Note 8).
6. Resuspended in prewarmed sporulation medium (25 ml final volume) and incubate at 37°C with agitation at 200 rpm during 2 hours (see Note 9). After 1 hour incubation add SYTOX Green to a final concentration of 7.5 nM and continue to incubate for the remaining hour.
7. Take 1 mL of sporulating bacterial suspension, place it into a 1.5 mL Eppendorf tube and spin down in a bench centrifuge at 4000 rpm for 3 min.
8. Discard supernatant and resuspend the pellet in 100 µL of filtered sporulation medium.
9. Add 1 uL of 1/10 dilution of 40 nm fluorescent beads to the suspension and gently mix. This solution is ready to be injected into the microfluidics chamber (see section 3.3).

3.1.2. Vegetatively growing B. subtilis

1. Streak HM675 bacteria from -80°C glycerol stock onto solid (plate) Luria-Bertani (LB) medium (i.e. agar plate) complemented with 2 µg/ml kanamycin.
2. Culture agar plates 24 hours at 20°C.
3. Select a single colony from plate and perform at least 5 serial dilutions with SMBS supplemented with CAA 0.02 mg/mL (see section 2.2) in sterile 50 ml falcon tubes (1/10 each in 5 ml final volume) and grow overnight at 30°C with agitation at 200 rpm.
4. The next morning measure the optical density (OD) at 600 nm and select dilution of bacteria exponentially growing (OD ~0.3-0.5). Further dilute to OD ~0.05 in SMBS (25 mL final volume in 250 mL flasks for optimal aeration) and incubate at 30°C with agitation at 200 rpm (see Note 10).
5. Measure OD at 1h intervals and when reaching OD~0.4-0.5, take 0.5 mL of bacterial suspension and place it into a 1.5 mL Eppendorf tube.
6. Add 50 µl of buffer phosphate 1 M pH 7.4. Mix gently.
7. Add 150 µl of fixation mix (see section 2.2). Mix gently until the solution is perfectly homogeneous.
8. Let the reaction proceed for 10 min. Next, place Eppendorf tube in an ice/water bath during 20 min.
9. Spin down bacteria in a bench centrifuge at 4000 rpm for 4 min (see Note 11).
10. Resuspend in 1 ml of SMM media.
11. Repeat steps 9 and 10 two times.
12. Spin down bacteria in a bench centrifuge at 4000 rpm for 4 min.
13. Discard supernatant and resuspend the pellet in 50 µl of SMM media.
14. Add 1 µL of 1/10 dilution of 40 nm fluorescent beads to the suspension and gently mix.

3.2. Coverslips and glass slides cleaning

1. Rinse coverslips and glass slides with acetone (See Note 12).
2. Rinse coverslips and glass slides with methanol.
3. Rinse coverslips and glass slides with water.
4. Place coverslips and glass slides in 1 M KOH solution and sonicate for at least 30 min.

5. Rinse coverslips and slides and store in water.
6. Before an experiment, dry coverslips and glass slides over an open flame to eliminate any remaining fluorescent contamination (see Note 13).

3.3. Sample mounting

3.3.1. Microfluidics device for imaging sporulating B. subtilis (see Note 14)

1. Microfluidics fabrication and assembly: Using a CO_2 laser cutter drill inlet/outlet ports through the 1mm thick glass slide. Using the same laser cutter design a parafilm mask containing a channel (Fig. 1B, see Notes 15-16).
2. Place parafilm mask sandwiched between a coverslip and a glass slide making sure each channel is properly aligned with a pair of inlet/outlet and heat to 90°C during 1 min (see Notes 17-18).
3. Wait 5 min for the microfluidics chamber to return to ambient temperature.
4. Mount microfluidics chamber into the microfluidics holder.
5. Connect inlets and outlets in the holder to the microfluidics chamber.
6. Fill the microfluidics channels with water and next with 0.01% (v/v) poly-L-Lysine and incubated for 5-10 min at room temperature (see Note 19).

3.3.2 Agarose pads for imaging vegetatively growing B. subtilis

1. Place a frame of double-side adhesive tape on a thick glass slide and extrude a ~5x5 mm square from its center (Fig. 1D-i-iii, see Note 20).
2. Add 50 µl of 2% melted agarose (diluted in SMM, melted at 90°C, see Notes 21-22) and spread on the centre of the square previously extruded.
3. Cover with a second glass slide (forming a 'sandwich') and add an additional weight (~ 400 g) on top of the second slide to ensure a flat agarose surface.
4. Keep on a horizontal position for 5 min under external pressure at room temperature (RT) to allow the agarose to solidify.
5. Remove gently the top slide when bacteria are ready to be deposited in the agarose pad (below).
6. Pipette 10 µl of vegetatively growing bacterial resuspension (see section 3.1.2) onto the agarose and let the deposited drop settle during 2 min to allow for spreading of the bacteria over the surface (see Note 23).

7. Remove the film of the second adhesive side of the tape and seal the pad with a clean coverslip. Apply homogeneous pressure to assure perfect sealing.

3.4. Imaging

3.4.1. SpoIIIE transporting DNA in sporulating B. subtilis

1. Mount microfluidics holder functionalized with poly-L-Lysine into the microscope and let stabilize during 30 min.
2. Rinse channels sequentially with water and sporulation medium (1 mL of each).
3. Inject 100 uL of sporulating bacterial resuspension (Fig. 1C-i, see section 3.1.1) and incubate during 5 min.
4. Apply a high flow force (~200 mL/s of sporulation buffer) to flatten cells against the surface (Fig. 1C-ii, see Notes 24-25).
5. Image DNA stained with SYTOX Green by exciting at 488 nm and collecting the emitted fluorescence with a 525 ± 25 nm bandpass filter (Fig. 1C-ii). Addapt exposure time and total number of frames depending on fluorescence intensity (usually 50-100 frames at 55 ms acquisition rate, see Note 26).
6. Before starting PALM imaging, adjust focal plane by using phase contrast images of bacteria and confirm the good focusing positioning by live fluorescence imaging on SYTOX Green-labeled DNA (see Note 27).
7. Turn on autofocus system to conserve the focal plane constant during the whole data collection process (see Note 28).
8. Switch emission filters before starting PALM imaging by using a 605 ± 35 nm bandpass filter and set the camera to 55 ms exposure time in frame transfer mode and set electronic gain to 300.
9. Acquire PALM images under continuous illumination with 561 nm laser (power density on the sample of ~ 0.2 kW/cm², see Note 29). Collect between 10,000 and 25,000 images (Fig. 1C-iii).
10. Employ pulses of the 405 nm laser for photo-activation of single emitters, starting with values for the pulse length and power of 5 ms and 10 W/cm^2, respectively and increase gradually (see Note 30).
11. Verify that all the fluorescent proteins have been activated and photobleached by sending a 405 nm laser pulse of the maximal intensity employed and with a

duration at least 10 times bigger than the last pulse employed. If all proteins have been properly photoactivated and bleached, a negligible number of stochastic events should be observed (see Note 31-32).

12. Slowly inject 100 µL of a 10 nM FM4-64 into the microfluidic chamber (Fig. 1C-iv).
13. Image bacterial membranes by exciting with the 561 nm laser and using the same emission filter as for PALM imaging (Fig 1C-iv, see Note 33).

3.4.2. ParB complexes organizing the B. subtilis origin of replication region

1. Mount agarose pad into microscope with the MicAO 3D-SR system and let stabilize during 30 min (Fig. 1D-iv).
2. Set MicAO 3D-SR parameters to enable astigmatic three-dimensional (3D) imaging: typically using 130nm RMS (see Note 34).
3. Record 3D calibration curves by selecting and imaging a single 100 nm TetraSpeck bead exciting with 561 nm laser and perform a scan of 2 µm of the sample stage along the optical axis (z) by steps of 50 nm (Fig. 1D-iv, see Note 34).
4. Before starting PALM imaging, adjust focal plane by using phase contrast images of bacteria.
5. Turn on autofocus system to conserve the focal plane constant during the whole data collection process (see Note 27).
6. Switch emission filters before starting PALM imaging of ParB-mEos2 by using a 605 ± 35 nm bandpass filter and set the camera to 50 ms exposure time in frame transfer mode and set electronic gain to 300.
7. Acquire PALM images under continuous illumination with 561 nm laser (power density on the sample of ~ 1.2 kW/cm², see Note 28). Collect between 10,000 and 25,000 images (Fig. 1D-v).
8. Employ continuous illumination with 405 nm laser for photo-activation of single emitters, starting with the lowest values available and increasing gradually to reaching a maximal power of 0.1 W/cm² (see Note 35).
9. Acquisition should be carried out until all mEos2 proteins are photo-activated and bleached (see Note 36).

3.5. Image reconstruction

3.5.1. 2D-PALM images of SpoIIIE transporting DNA in sporulating B. subtilis

1. Use MTT-analysis software ***(19)*** to detect the coordinates of localization of SpoIIIE-EosFP-specific and bead-specific fluorescence events from acquisition performed in section 3.4.1.
2. Fed the coordinates detected by MTT to PALMCBS ***(13, 21)***.
3. Select fiducial marks (beads) and perform lateral drift correction (see Note 37) of single-molecule detected events.
4. Select epi-fluorescence images of stained DNA and membranes (Fig. 2A) or bright field images (Fig. 2B) to overlay with SMLM rendered image.
5. Select SMLM rendering mode: density map (Fig. 2A) or pointillist (Fig. 2B) and obtain final images (see Notes 38-39).

3.5.2. 3D-PALM images of ParB complexes organizing the B. subtilis origin of replication region

1. Obtain 3D calibration curves using PALMCBS: employ the axial scan of step 3 from section 3.4.2 to fit the PSF of the bead with an elliptical 2 dimensional Gaussian function for each z position and calculate the respective x-y widths (w_x and w_y). Plot the obtained x-y widths as a function of z position and fit with third order polynomial function to produce the calibration curves $w_x(z)$ and $w_y(z)$ that will allow to estimate the axial position of each single-molecule event detected in the next steps (see Note 34).
2. Obtain 3D single-molecule localizations using Rapid STORM ***(20)***.
3. Use Rapid STORM to detect the coordinates of localization of ParB-mEos2 and bead-specific fluorescence events from acquisition performed in section 3.4.2.
4. Feed the coordinates detected by Rapid STORM to PALMCBS ***(13, 21)***.
5. Select fiducial marks (beads) and perform axial and lateral drift correction of single-molecule detected events (see Note 40).
6. Select bright field images to overlay with SMLM rendered image.
7. Select SMLM rendering mode: volumetric map and obtain final images (Fig. 2C, see Note 41).

4. Notes

1. Do not autoclave solution A as precipitation of different components may occur.
2. SMBS media should be prepared fresh for each experiment and stored no longer than 24 hours.
3. For experiments with *B. subtilis* strains in BS168 background Tryptophan should be added to the SMBS media to a final concentration of 0.02 mg/mL.
4. SMBS solution should be prewarmed to room temperature when performing dilutions of growing bacteria.
5. Microscope setup (Fig. 1A): three lasers with excitation wavelengths of 405 nm, 488 nm and 561 nm are expanded to a beam diameter of 2mm and combined into a single co-linear beam using dichroic mirrors (DM1). Two achromatic lenses (L1a & b) are used to expand the excitation beam and to obtain a homogeneous illumination over fields of view as large as 40 x 40 μm^2. The lasers are focused by L1b, located near the back port of the microscope, and directed by dichroic mirror DM2 to the back focal plane of a 100x Plan-Apo oil objective (OBJ, NA = 1.4) mounted on a z-direction piezoelectric stage (PZ). A motorized stage (MS) is used to translate the sample perpendicularly to the optical axis. Fluorescence from the sample is collected by the objective, separated from laser light by dichroic mirrors DM2 and DM3 and focused by the tube lens (TL) on the microscope original imaging plane (IP). A pair of achromatic relay lenses arranged as a telescope (L2a & b) is used to form an image on the EMCCD camera sensor (C1) while increasing the total magnification of the system (effective pixel size of 115 nm). A motorized filter wheel (FW) placed between the two lenses allows for the selection of the fluorescence emission filter. The emission intensity of each laser is controlled individually using a USB-DAQ device (National Instruments). Control software for the lasers and the filter wheel is written in LabView 2010 (National Instrument, France).

6. Active autofocus system (Fig. 1A): in a separate path from the other three lasers, a linearly polarized 1064 nm IR beam from an Ytterbium fiber laser is expanded twice (L3a & b) and passes through an optical separator formed by a polarized beam splitter (PBS) and a quarter wave plate (QWP). The beam is directed towards the objective lens by dichroic mirror DM3. Depending on the sample and the position of the plane imaged by the objective, the distance between L3a and L3b can be modified to ensure that the IR

beam is always focused at the glass/sample interface. Part of the IR beam is reflected by the sample, collected by the objective and redirected towards the PBS following the same path than the incident beam. Due to a change in polarization introduced by reflection at right angles, the reflected beam is redirected by the PBS towards lens L4 and imaged on a CCD detector (C2). A half-wave plate (HWP) is used to manually adjust the intensity of the incident beam. Control software for the autofocus was written in LabView 2009, using the PID and Fuzzy Logic Toolkit. A feedback loop between the CCD detector (C2) and the piezo stage (PZ) makes sure that the sample remains in focus at all times during the PALM acquisition (resolution of ~5-10 nm over hours).

7. When imaging proteins with low expression levels such as SpoIIIE (~6-8 hexamers located at the sporulating septum), tubes holding bacterial cells should be protected with aluminum foil to avoid light exposure at all times .

8. In our hands, the sporulation efficiency was increased when leaving a very slight supernatant of LB20% (~0.25-0.5 mL).

9. In some cases, sporulation is achieved after longer incubations times (up to 3 hours). However, if after 3-4 hours the sporulation efficiency is below 30-40% this should be an indicative that the optimal experimental conditions have not been reached.
10. CAA are not added to final working solution to slow down growth rate and to diminish bacteria autofluorescence background.
11. If the initial concentration of bacteria was not high enough, higher centrifugation speeds may be used.
12. For all steps involving coverslips and slides cleaning, carefully rinse each side using tweezers to hold the coverslips and glass slides to avoid contamination.
13. When using the flame, do not expose the coverslips to the hottest portion of the flame as too much heat will either break them instantly or fragilize them representing a risk of breaking during sample mounting.
14. A detailed guide to assembly a similar microfluidics chamber to the one presented here can be found at ***(22)***. The microfluidics system introduced in this protocol is ideally suited for SMLM microscopy, as it allows for the sequential imaging of fluorophores thus avoiding chromatic aberrations and channel bleed-through, is highly stable over long

periods of time, and by exchanging the surface functionalizing polymers allows for time-lapse imaging of living cells.

15. The detailed plans for microfluidics design are available under request.
16. A thick glass slide was used to ensure a better rigidity of the chamber and to avoid deformation when approaching the objective lens.
17. Use the steel blocks of a heat dry bath to compress the microfluidic chamber.
18. An indicative that the microfluidics chamber will remain stably bound by parafilm is to verify that after heating during 1 min at 90 it becomes transparent (when cooling down it should return to its usual semi-transparent opaque appearance).
19. Alternatively poly-L-Lysine can be replaced by chitosan, a linear polysaccharide composed of randomly distributed β-(1–4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit) in which, under continuous media flow, bacteria are able to grow ***(21)***.
20. A thick glass slide was used to ensure a better rigidity of the agarose pad assembly and to avoid deformation when approaching the objective lens.
21. The agarose stocks should not be reused after heating to 90°C as agarose-dependent autofluorescence can be observed and give false positive localization events.
22. The high concentration of agarose was used to improve the stability of the pad during SMLM experiments.
23. Do not allow agarose pad to dry as the surface becomes irregular and bacteria will not be flat on the surface. This is critical when performing 3D PALM imaging.
24. The ratio between bacteria and beads should be adjusted to obtain an optimal density of 5-15 beads per field of view. In average, 100-150 bacteria per field of view could be imaged at high resolution.
25. When using poly-L-lysine-coated surfaces, each experiment should be performed in a new channel, and measurements should be performed within the first 15 min after injection of cells into the channel to avoid any possible surface-immobilization defect.
26. It is ideal to obtain a well contrasted image of bacterial DNA if further cell segmentation steps are planned. For the initial segmentation DNA images are more robust than membrane images since, DNA stain images of cells in physical contact will show a gap in fluorescence signal between them, whereas the membrane stain of both cells will overlap at the contact point, making segmentation more difficult.

27. The time of SYTOX Green-labeled DNA imaging to obtain final focus positioning should be limited to a minimal to avoid DNA damage and cell death.
28. At the beginning of each experiment, a calibration is carried out to ensure that the intensity of the IR reflection varies linearly over a course of ~600 nm around the plane imaged by the objective. When the acquisition starts, the intensity of the IR reflection is used as reference and axial drift is corrected by adjusting the position of the objective. The focal plane feedback has to be nm accurate. The one employed for this experiments guaranteed less than 5-10 nm axial drift in 2 to 3 hours.
29. The 561 nm laser power was optimized for the detection of single photoactivatable proteins while preventing activation induced by the readout laser and cells photodamage.
30. The length and power of the 405 nm laser pulses should be slowly increased during the course of the experiment in order to maintain the density of activated fluorophores constant.
31. For initial characterization of unknown structures, this final step employing a long 405 nm laser pulse will allow to refine both excitation and activation laser intensities and pulse durations as well as the total number of frames to be cumulated to obtain good quality images.
32. When imaging new cell lines or strains, a control with wild-type cell line (i.e. devoid of any genetically introduced fluorescent probe) allows to verify the absence of high background levels and detection of false positive events associated to imaging conditions (e.g. emission or blinking of naturally occurring fluorescent molecules in the cell that may emit at similar wavelengths than that of the photo-activatable probe).
33. Using the same emission filter prevents chromatic aberration between PALM images and membrane images. In the case of membrane proteins, as SpoIIIE, this can be particularly useful when the protein localization with respect to the bacterial membrane is investigated.
34. The most important factor of a high quality calibration curve is the difference between the X and Y width, which ideally forms a straight line. The slope of this line is indicative of the Z localization precision, which can be tuned by the amount of astigmatism applied by the deformable mirror – the bigger the amplitude of astigmatism, the steeper the slope, and thus the more precise Z localization. For more details on the use of MicAO 3D-SR system and calibration see utilization notes in ***(23, 24)***.

35. The intensity of the 405 nm laser should be modified during the course of the experiment to maintain the density of activated fluorophores constant while ensuring that only one protein was activated at a time in a single diffraction-limited spot. Higher 405 nm laser powers than 0.1 kW/cm^2 may lead to non-specific fluorescent events (located inside and outside the bacterial cells) and thus to false single-molecule detections in the ulterior image analysis.
36. In our hands, for fixed *B. subtillis* containing ParB-mEos2, the large majority of proteins were photo-activated and bleached before 15000 frames were registered. Note that chemical fixation procedures may deteriorate the photophysical properties of fluorescent proteins and even abolish completely fluorescence emission in certain cases. Further optimization may be required in such cases.
37. Lateral drift correction over the full acquisition period is assessed by plotting the trajectories of fluorescent beads in x and y coordinates over time as described in ***(13, 21)***. For this procedure, only beads detected during the entire acquisition (~15-20 min) should be employed. Trajectory curves are smoothed by a Stavinsky-Golay filter and overlaid by minimising the distance between each trajectory using the first selected bead as reference. The origin was calculated by averaging the trajectories over the first 100 images, ensuring the drift was equal to zero at t = 0 min. At least 5 beads should be employed to correct the trajectory of selected regions containing one or two cells (~50 x 50 pixels). The quality of the drift correction is estimated by subtracting the reference to all the trajectories and calculating the standard deviations along x and y. Experiments having values of drift correction precision above 10 nm (when using poly-L-Lysine) should be discarded.
38. SMLM rendering using density plots: in this type of rendering the localization coordinates are represented as a 2D histogram in which the intensity is proportional to the number of localizations in a given pixel with a user-defined size (10 nm in figure 2A). This representations although visually attractive and useful to observe the complex of interest respect to other cellular components, can mask important structural details (e.g.. low density of labelling can be interpreted as substructural features) and it is not fully reliable to evaluate the quality of SMLM image obtained.
39. SMLM rendering using pointillist representation: in this kind of representation the unweighted positions of localized molecules are depicted as dots or markers. This representation may highlight structural details and may be useful to employ when evaluating

different clusterization algorithms or when performing multi-color SMLM imaging. A drawback to this kind of representation arises when studying highly dense or small structures where superposing independent events can not be visualized and thus the information concerning the relative number of single-molecules belonging to a complex is lost.

40. Fluorescent beads are used to correct for drift in all three directions: on agarose pad, lateral and axial drifts precisions were 10 ± 5 nm and 25 ± 10 nm respectively. Samples with abnormal drift or lesser drift precision correction should be discarded.

41. 3D SMLM rendering using volumetric map representation: After segmenting bacteria by using bright field images and MicrobeTracker ***(25)*** PALMCBS allows to obtain volumetric representations of objects by grouping together detected single-molecule events. For the example depicted in figure 2C a binarized 3D density map was computed by dividing the bacteria volume into voxels of 30 nm along the x-y axis and 50nm along the z axis. These values were chosen according to the lateral and axial resolution previously reported for 3D-PALM experiments based on astigmatism detection. The value of the voxels containing at least one single-molecule fluorescent event was set to 1. The value of all the other voxels were set to 0. Using the binary density map and a clustering algorithm, a list of clusters was produced and the number of fluorescent events associated to each cluster was calculated. Sub-clusters containing less than 5 detections were rejected to avoid non-specific localization events and mis-localizations artifacts.

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Figure legends

Figure 1. A. PALM microscope components and setup (see Notes 5-6). Lasers: 405 nm, 488 nm, 561 nm and 1064 nm. Dichroic mirrors (DM1, DM2 and DM3). Achromatic lenses (L1a, L1b, L2, L2a, L2b, L3a and L3b). 100x Plan-Apo oil objective (OBJ, NA = 1.4). Piezoelectric stage (PZ). Motorized stage (MS). Acousto-Optic Tunable Filters (AOTF). Tube lens (TL). Polarized beam splitter (PBS). Half-wave plate (HWP). Quarter wave plate (QWP). Microscope original imaging plane (IP).MicAO 3D-SR system. EMCCD camera sensor (C1). CCD detector (C2). **B. Micro-fluidic chamber assembly.** A a coverslip and a 1mm thick glass slide containing a three-way inlet and single outlet ports were sealed together by a parafilm mask melted at 90°C during 1 min. **C. Sequential SMLM imaging procedure in microfluidic chamber.** (i) The microfluidic chamber was filled with a 0.01% (v/v) solution of poly-L-Lysine and incubated for at least 5 min at room temperature. After washing with sporulation media, 100 µL of a concentrated solution of bacterial cells along with fiducial marks were injected and let settle onto the coated surface. (i-ii) A high flow force was applied by pumping sporulation medium to rinse the channel, wash away unattached bacteria and ensure that attached bacteria laid completely flat on the surface. (ii) DNA was imaged by epi-fluorescence microscopy, and (iii) SpoIIIE was imaged by SMLM. (iv) Finally, the FM4-64 membrane staining agent was injected allowing for bacterial membrane detection by epi-fluorescence. **D. SMLM imaging of bacteria in agarose pads.** (i) A rectangular double-side adhesive tape was placed on thick glass slide with a square of ~5x5 mm extruded from its center. (ii) Melted agarose was added to create an adhering surface for the bacteria. (iii) Bacterial cells mixed with fiducial marks, were deposited on agarose and the pad was sealed with a clean coverslip and the sample was finally mounted into the microscope stage (iv-v) Sequential imaging of bacterial membrane and SpoIIIE (iv) Epi-fluorescence image of the cell membrane was collected by exciting at 561 nm. (v) SMLM images were collected by using continuous excitation with a 561 nm laser and by applying regular pulses of photo-activation with a 405 nm laser. Figures in panels A, B and C were reproduced with permission from ***(13, 21)***.

Figure 2. Different SMLM image reconstruction representations. A. Probability density representation (see Note 38) of SpoIIIE localization in sporulating cells. Stained DNA and bacterial membranes are false colored violet and red respectively. **B.** Pointillist representation (see Note 39) of SpoIIIE (green dots) in sporulating cells overlaid with a bright field image of the

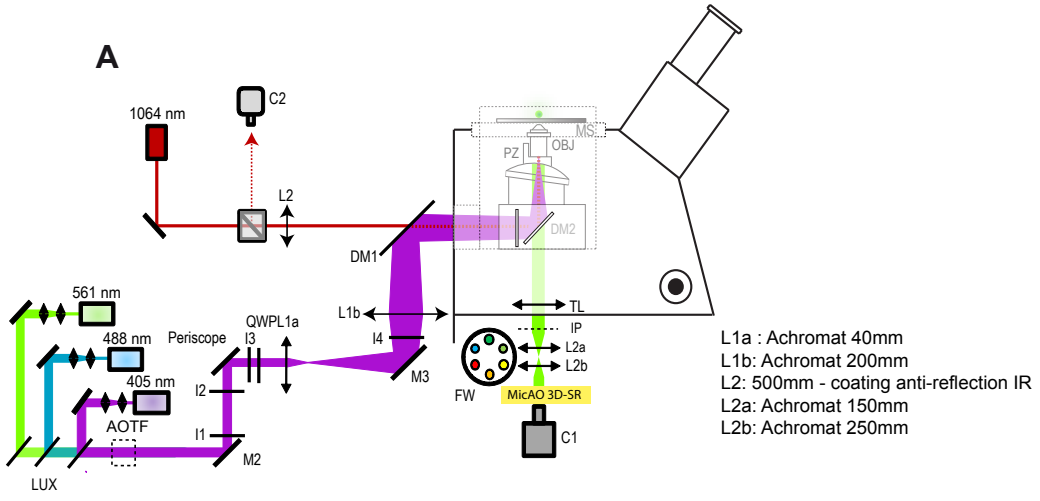
membrane (white). Each green dot represents a single fluorescent event detected in a single frame during the SMLM acquisition. **C.** 3D-PALM images of origins domains labeled with ParB-mEos2 (pink, see Note 41). The cell volume was reconstructed from bright field images (dark grey contour) and an approximative localization within that volume of the bacterial nucleoid is indicated by the dashed black line (nucleoid). Figures in panels A, B and C were reproduced with permission from ***(13, 17)***.

References

1. Rust MJ, Bates M, and Zhuang X (2006) Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). Nat Methods 3:793–795
2. Hess ST, Girirajan TPK, and Mason MD (2006) Ultra-high resolution imaging by fluorescence photoactivation localization microscopy. Biophys J 91:4258–4272
3. Betzig E, Patterson GH, Sougrat R, et al (2006) Imaging intracellular fluorescent proteins at nanometer resolution. Science 313:1642–1645
4. Heilemann M, Linde S van de, Schüttpelz M, et al (2008) Subdiffraction-resolution fluorescence imaging with conventional fluorescent probes. Angew Chem Int Ed Engl 47:6172–6176
5. Heilemann M, Linde S van de, Mukherjee A, et al (2009) Super-resolution imaging with small organic fluorophores. Angew Chem Int Ed Engl 48:6903–6908
6. Ptacin JL, Lee SF, Garner EC, et al (2010) A spindle-like apparatus guides bacterial chromosome segregation. Nat Cell Biol 12:791–798
7. English BP, Hauryliuk V, Sanamrad A, et al (2011) Single-molecule investigations of the stringent response machinery in living bacterial cells. Proc Natl Acad Sci U S A 108:E365–73
8. Wang W, Li G-W, Chen C, et al (2011) Chromosome organization by a nucleoid-associated protein in live bacteria. Science 333:1445–1449
9. Badrinarayanan A, Reyes-Lamothe R, Uphoff S, et al (2012) In vivo architecture and action of bacterial structural maintenance of chromosome proteins. Science 338:528–531
10. Biteen JS, Goley ED, Shapiro L, et al (2012) Three-dimensional super-resolution imaging of the midplane protein FtsZ in live Caulobacter crescentus cells using astigmatism. Chemphyschem 13:1007–1012
11. Uphoff S, Reyes-Lamothe R, Garza de Leon F, et al (2013) Single-molecule DNA repair in live bacteria. Proc Natl Acad Sci U S A 110:8063–8068
12. Buss J, Coltharp C, Huang T, et al (2013) In vivo organization of the FtsZ-ring by ZapA and ZapB revealed by quantitative super-resolution microscopy. Mol Microbiol 89:1099–1120
13. Fiche J-B, Cattoni DI, Diekmann N, et al (2013) Recruitment, assembly, and molecular architecture of the SpoIIIE DNA pump revealed by superresolution microscopy. PLoS Biol 11:e1001557
14. Holden SJ, Pengo T, Meibom KL, et al (2014) High throughput 3D super-resolution microscopy reveals Caulobacter crescentus in vivo Z-ring organization. Proc Natl Acad Sci U S A 111:4566–4571
15. Stracy M, Lesterlin C, Garza de Leon F, et al (2015) Live-cell superresolution microscopy reveals the organization of RNA polymerase in the bacterial nucleoid. Proc Natl Acad Sci U S A 112:E4390–9
16. Jacq M, Adam V, Bourgeois D, et al (2015) Remodeling of the Z-Ring Nanostructure during the Streptococcus pneumoniae Cell Cycle Revealed by Photoactivated Localization Microscopy. MBio 6
17. Marbouty M, Le Gall A, Cattoni DI, et al (2015) Condensin- and Replication-Mediated Bacterial Chromosome Folding and Origin Condensation Revealed by Hi-C and Super-resolution Imaging. Mol Cell 59:1–15
18. Sterlini JM and Mandelstam J (1969) Commitment to sporulation in Bacillus subtilis and its relationship to development of actinomycin resistance. Biochem J 113:29–37
19. Sergé A, Bertaux N, Rigneault H, et al (2008) Dynamic multiple-target tracing to probe

spatiotemporal cartography of cell membranes. Nat Methods 5:687–694
20. Wolter S, Löschberger A, Holm T, et al (2012) rapidSTORM: accurate, fast open-source software for localization microscopy. Nat Methods 9:1040–1041
21. Cattoni DI, Fiche J-B, Valeri A, et al (2013) Super-resolution imaging of bacteria in a microfluidics device. PLoS One 8:e76268
22. Cheng W (2015) Mechanisms of HCV NS3 helicase monitored by optical tweezers. Methods Mol Biol 1259:229–255
23. Clouvel G, Jasaitis A, and Levecq X (2015) 3D dual-color PALM/dSTORM imaging of centrosomal proteins with nanometric resolution using MicAO 3DSR.
24. Jasaitis A, Clouvel G, and Levecq X (2015) Deep 3D PALM/STORM imaging MicAO 3DSR - the key to combining depth and highest resolution.
25. Sliusarenko O, Heinritz J, Emonet T, et al (2011) High-throughput, subpixel precision analysis of bacterial morphogenesis and intracellular spatio-temporal dynamics. Mol Microbiol 80:612–627

Figure 1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L2
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MS
PZ
OBJ
DM2
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488 nm
405 nm
AOTF
LUX
Periscope
QWPL1a
I3
I2
I1
M2
L1b
I4
M3
TL
IP
L2a
L2b
FW
MicAO 3D-SR
C1
L1a : Achromat 40mm
L1b: Achromat 200mm
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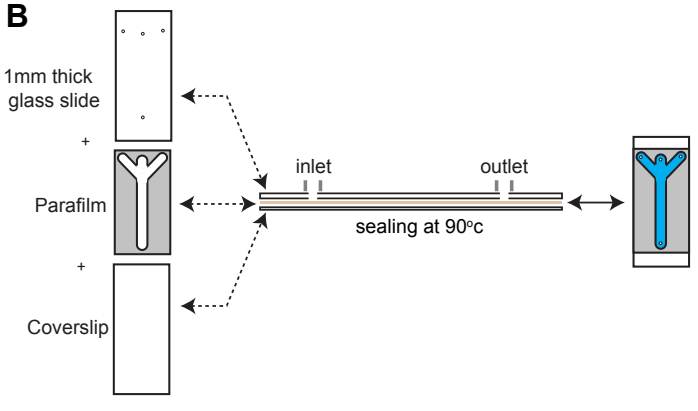
L2b: Achromat 250mm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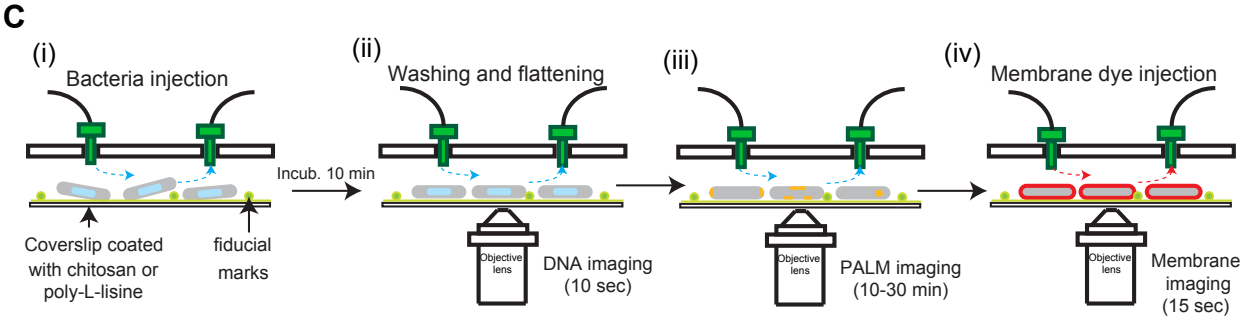
+
Parafilm
+
Coverslip
inlet
outlet
sealing at 90°c


C
(i)
Bacteria injection
Coverslip coated with chitosan or poly-L-lisine
fiducial marks
Incub. 10 min
(ii)
Washing and flattening
Objective lens
DNA imaging (10 sec)
(iii)
PALM imaging (10-30 min)
(iv)
Membrane dye injection
Membrane imaging (15 sec)

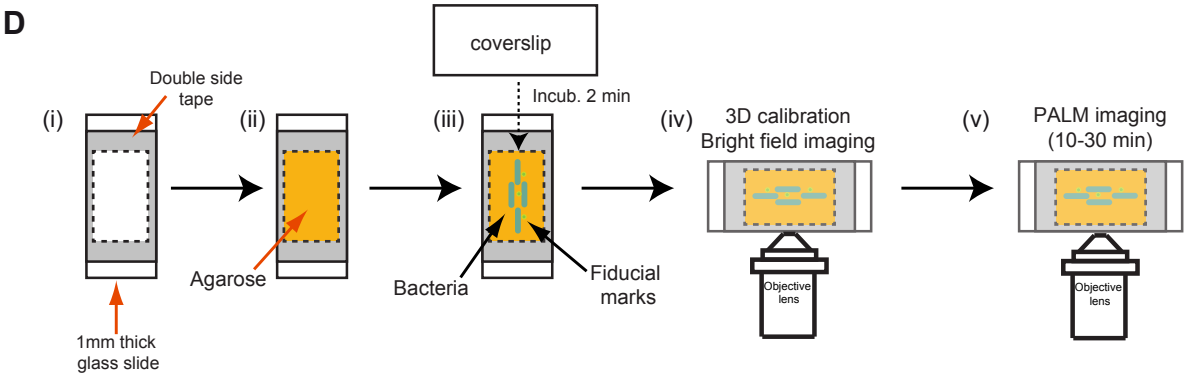
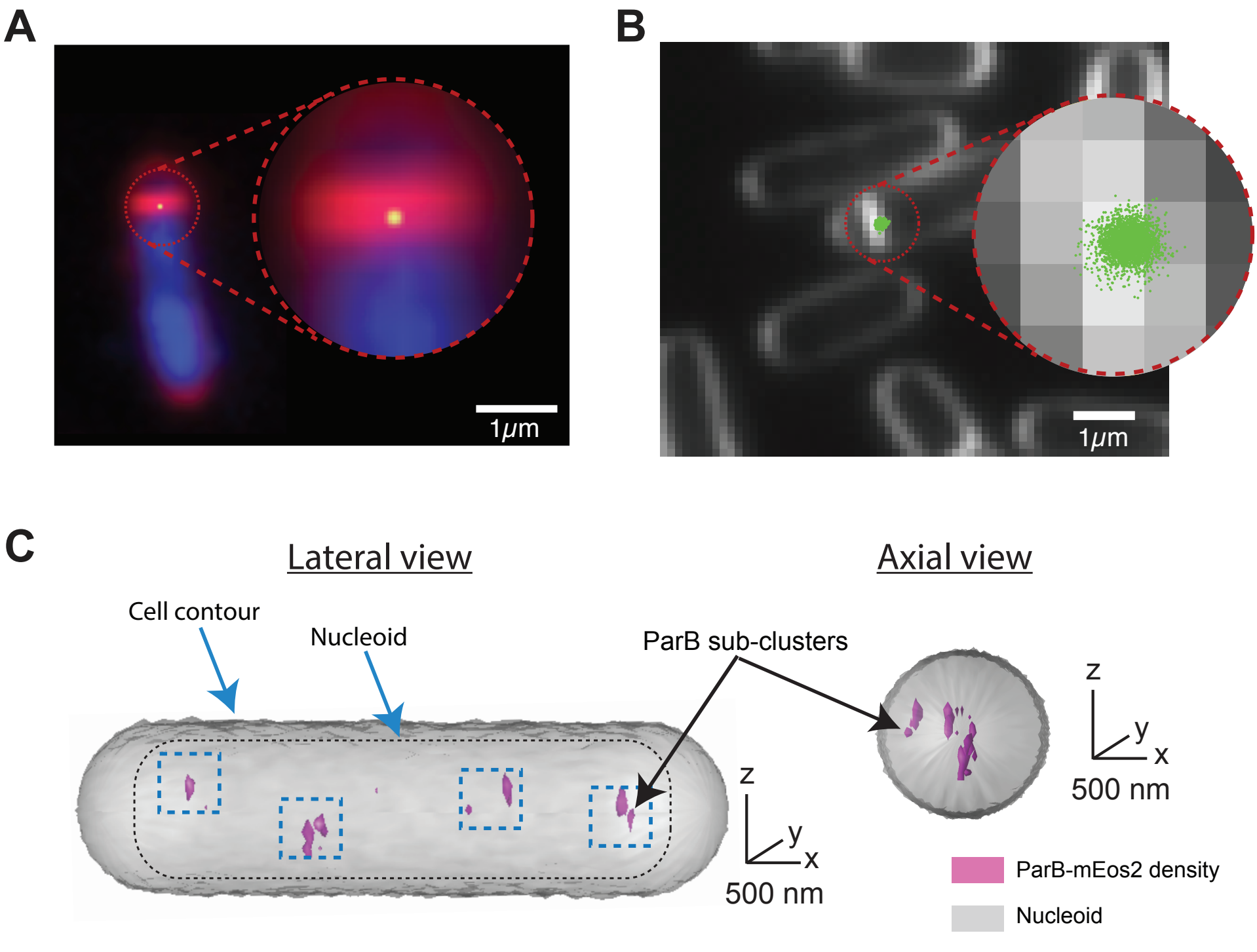

D
(i)
Double side tape
1mm thick glass slide
(ii)
Agarose
coverslip
Incub. 2 min
(iii)
Bacteria
Fiducial marks
(iv)
3D calibration
Bright field imaging
Objective lens
(v)
PALM imaging (10-30 min)
Objective lens

Figure 2


A
1μm
B
1μm
C
Lateral view
Axial view
Cell contour
Nucleoid
ParB sub-clusters
z
y
x
500 nm
z
y
x
500 nm
ParB-mEos2 density
Nucleoid