Chapter 19

Visualizing *Bacillus subtilis* During Vegetative Growth and Spore Formation

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Abstract

Bacillus subtilis is the most commonly used Gram-positive bacterium to study cellular processes because of its genetic tractability. In addition, during nutrient limitation, *B. subtilis* undergoes the development process of spore formation, which is among the simplest examples of cellular differentiation. Many aspects of these processes have benefited from fluorescence microscopy. Here, we describe basic wide-field fluorescence microscopy techniques to visualize *B. subtilis* during vegetative growth, and the developmental process of sporulation.

Key words *Bacillus subtilis*, Vegetative growth, Sporulation, Fluorescence microscopy, Time-lapse microscopy

1 Introduction

Bacillus subtilis is a rod-shaped soil bacterium that grows and divides through binary fission. When nutrients become limited, it undergoes a developmental process that results in the formation of a dormant spore [1]. The highly resistant spore allows the bacteria to survive extreme environmental conditions including heat, desiccation, UV and γ-radiation, and presence of antibiotics and other toxic chemicals [2]. When nutrients become available, the spores can germinate and resume exponential growth [3]. This double life-style and the ease with which genetic, biochemical, and cytological analysis can be carried out in this organism make *B. subtilis* an ideal system to study a variety of cellular processes, such as gene regulation, chromosome dynamics, morphogenesis, and cell fate determination.

The application of fluorescence microscopy to the study of prokaryotes has revealed that bacteria possess a highly organized internal architecture [4]. Even at a time in which super-resolution imaging [5] and microfluidic technologies are becoming more and more common [6–8], wide-field fluorescence microscopy using

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simple glass slides still remains a powerful tool and workhorse in the field. In this chapter, we describe how we routinely grow *B. subtilis* in liquid culture, and how we prepare slides for snapshot and time-lapse imaging in a very simple experimental setup. Although it is a popular model organism, compared to other model bacteria like *Escherichia coli* and *Caulobacter crescentus*, *B. subtilis* appears to require more oxygen for growth and most importantly, for microscopy. In this chapter, we specifically emphasize the importance of aeration of liquid cultures and the use of open agarose pads for time-lapse microscopy to maintain adequate oxygen for balanced growth of *B. subtilis* in liquid and on slides. Additionally, we have observed that photobleaching and phototoxicity seem to be a bigger challenge when imaging *B. subtilis*, perhaps related to its requirement for oxygen. Therefore, the use of a low-fluorescence background growth medium and a systematic image acquisition optimization are especially important when visualizing *B. subtilis*.

2 Materials

Media components are prepared in Milli-Q water (ddH_2O) unless otherwise stated. Where indicated, liquid media are sterilized by autoclaving for 30 min in liquid cycle, or by filtering through a 0.22-μm syringe-driven filter or a bottle-top filter. Glassware is sterilized by autoclaving for 30 min in dry cycle. Sterile media components and glassware are stored at room temperature unless otherwise specified.

2.1 Media and Equipment for the Growth of B. subtilis

1. Make regular LB agar plates, or plates containing one of the following antibiotics if needed: 5 μg/ml chloramphenicol; 1 μg/ml erythromycin plus 25 μg/ml lincomycin (MLS); 10 μg/ml kanamycin; 0.4 μg/ml phleomycin; 100 μg/ml spectinomycin; 10 μg/ml tetracycline (*see* **Note 1**).
2. Defined rich casein hydrolysate medium (CH medium) [9] component CHI + II: 10 mg/ml casein hydrolysate (Neogen #7229A), 4.7 mg/ml l-glutamate sodium salt monohydrate, 3.2 mg/ml l-asparagine monohydrate, 2.5 mg/ml l-alanine, 2.72 mg/ml potassium phosphate monobasic anhydrous (KH_2PO4), 2.68 mg/ml ammonium chloride (NH_4Cl), 1.1 mg/ml sodium sulfate (Na_2SO_4), 1 mg/ml ammonium nitrate (NH_4NO_3), and 0.01 mg/ml ferric chloride 6-hydrate ($FeCl_3 \cdot 6H_2O$); autoclaved.
3. CH medium component CHIII: 0.66 mg/ml calcium chloride dehydrate ($CaCl_2 \cdot 2H_2O$), and 1.21 mg/ml magnesium sulfate anhydrous ($MgSO_4$); autoclaved.

4. CH medium component CHIV: 1.67 mg/ml manganese sulfate monohydrate ($MnSO_4 \cdot H_2O$); autoclaved.
5. CH medium component CHV: 2 mg/ml l-Tryptophan; filtered and distributed in 10 ml aliquots in 15 ml conical tubes. Store at 4 °C.
6. S750 minimal medium [10] component deionized water (dH_2O); autoclaved (*see* **Note 2**).
7. S750 minimal medium component 10× S750 salt: 104.7 mg/ml 4-morpholinepropanesulfonic acid (MOPS free acid), 13.2 mg/ml ammonium sulfate [$(NH_4)_2SO_4$], and 6.8 mg/ml potassium phosphate monobasic (KH_2PO_4); autoclaved.
8. S750 minimal medium component 100× metals: 200 mM $MgCl_2$, 70 mM $CaCl_2$, 5 mM $MnCl_2$, 0.1 mM $ZnCl_2$, 0.1 mg/ml thiamine HCl, 0.002 N HCl, 0.5 mM $FeCl_3$; filtered and distributed in 10 ml aliquots in 15 ml conical tubes. Wrap tubes in foil and store at 4 °C.
9. S750 minimal medium component 1 M glutamic acid potassium salt: adjust to pH 7.0 using potassium hydroximate (KOH); filtered.
10. S750 minimal medium component 50% glucose; filtered. If a slower growth rate is needed, 50% sorbitol can be used instead of glucose.
11. Glass culture tubes (18 × 150 mm, VWR 47729-583), autoclaved; and roller drum in 22 °C incubator. Similar tubes or rolling/shaking systems can be used to grow 5 ml of liquid culture with aeration.
12. 250 ml baffled flasks, autoclaved.
13. Temperature-controlled shaking waterbath that can accommodate 250 ml flasks. Alternatively, temperature controlled air-shakers can be used.
14. Spectrophotometer to monitor optical density (OD_{600}).

2.2 Components for Snapshot Imaging

1. Standard glass slides, and cover slips (glass thickness 0.17 mm/No.1.5).
2. (Optional) Red membrane stain FM4-64 (*N*-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino) phenyl) hexatrienyl) pyridinium dibromide) (Life Technologies T-3166) (100×): 0.15 mg/ml in ddH_2O; protect from light and store at 4 °C. This dye can be visualized using Chroma's ET 49008 single band filter set (*see* **Note 3**).
3. (Optional) Blue DNA stain DAPI (4′,6-diamidino-2-phenylindole) (Life Technologies D-1306) (100×): 0.2 mg/ml in ddH_2O; protect from light and store at 4 °C. This dye

can be visualized using Chroma's ET 49000 single band filter set (*see* **Note 3**).

4. (Optional) Blue membrane stain TMA-DPH (1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate) (Life Technologies, T-204) (100×): 5 mM in DMSO; protect from light and store at 4 °C. This dye can be visualized using Chroma's ET 49000 single band filter set (*see* **Note 3**).
5. Molecular grade agarose.
6. Microwave oven.
7. Kimwipes.
8. Ethanol in squirt bottle.
9. Nikon Ti microscope equipped with a Plan Apo 100×/1.4 N.A., phase-contrast oil objective, a CoolSnapHQ^2 camera, Nikon Ti-S-ER motorized stage, filter cubes for GFP (Chroma 49002), YFP (Chroma 49003), CFP (Chroma 49001), DAPI (Chroma 49000), Texas Red (Chroma 49008), and a Lumencor Spectra X light engine. Similar microscopes can be used.

2.3 Components for Time-Lapse Imaging

1. Molecular grade agarose.
2. Microwave oven.
3. 60 × 15 mm petri dish (Becton Dickinson Labware 351007).
4. Scalpel.
5. Glass bottom dish (Willco Wells 50/40 mm Glass Thickness 0.17 mm/No.1.5 HBSt-5040).
6. Humidified stage-top incubator (TC-MIS; Bioscience Tools) and objective heater (Bioptechs). Other humidified, temperature-controlled environmental chambers for microscopes can be used.
7. Microscope (*see* **item 9** in Subheading 2.2) with a Well Plate Holder stage (TI-SH-W; Nikon) for stage-top incubator. Other inverted microscopes can be used.

2.4 Components for Visualizing Sporulating Cells

1. Sporulation resuspension medium [9] component ddH_2O, autoclaved.
2. Sporulation resuspension medium component Solution A: 0.089 mg/ml ferric chloride 6-hydrate ($FeCl_3 \cdot 6H_2O$), 0.83 mg/ml magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$), 1.98 mg/ml manganese chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$); filtered and dispensed into 10 ml aliquots in 15 ml conical tubes. Wrap with foil and store at 4 °C.
3. Sporulation resuspension medium component Solution B: 13.4 mg/ml ammonium chloride (NH_4Cl), 2.65 mg/ml sodium sulfate (Na_2SO_4), 1.7 mg/ml ammonium nitrate

(NH_4NO_3), 2.43 mg/ml potassium phosphate monobasic anhydrous (KH_2PO_4); Dissolve in 80% volume, adjust to pH 7.0 using 1 N NaOH, add ddH_2O to final volume; autoclaved.

4. Sporulation resuspension medium component Solution C: 63.6 mg/ml l-glutamic acid sodium salt monohydrate, which represents 5% l-glutamic acid; autoclaved.
5. Sporulation resuspension medium component Solution D: 3.68 mg/ml calcium chloride dehydrate ($CaCl_2 \cdot 2H_2O$); autoclaved.
6. Sporulation resuspension medium component Solution E: 120.4 mg/ml magnesium sulfate anhydrous ($MgSO_4$); autoclaved.
7. Bench top centrifuge for spinning 50 ml conical tubes.
8. Components for snapshot imaging, same as **items 1–9** in Subheading 2.2.

3 Methods

3.1 Exponential Growth of Cells

1. Streak out the *B. subtilis* strain of interest on an LB agar plate containing the appropriate antibiotics and incubate at 37 °C overnight (*see* **Note 4**). Use a different temperature if the strain has a specific requirement.
2. If the colonies are visible the next morning, take the plate out and leave at room temperature during the day to prevent over growth (*see* **Note 5**).
3. In the evening, prepare 100 ml of complete CH medium using the individual components. The doubling time for wild-type PY79 strain growing in CH is ~35 min (*see* **Note 6**). For complete CH medium, combine 94 ml of CHI + II, 4 ml of CHIII, 1 ml of CH IV, and 1 ml of CHV in a sterile bottle. If minimal medium is used instead of CH medium, make 100 ml of S750 medium by combining 86 ml of autoclaved dH_2O, 10 ml of 10× S750 salt, 1 ml of 100× metals, 2 ml of 1 M glutamic acid potassium salt, and 2 ml of 50% glucose or sorbitol in a sterile bottle. The doubling time for wild-type PY79 strain growing in S750 glucose is ~48 min and in S750 sorbitol is 80–100 min.
4. Set up a starter culture by inoculating a single colony into 5 ml of growth medium (complete CH medium or S750 minimal medium), vortex gently to disperse the colony (*see* **Note 7**). Make a 1–5 dilution of the inoculum by transferring 1 ml of the inoculum into 4 ml of fresh medium. Put both tubes in roller drum and roll overnight at 22 °C (*see* **Note 8**).

5. In the next morning, measure the optical density (OD_{600}) of both cultures and use the starter culture that is between 0.2 and 0.6. Dilute the culture in 25 ml of fresh growth medium prepared the previous day (*see* **step 3**) in a 250 ml baffled flask to an OD_{600} of 0.02 (*see* **Note 9**).
6. Put the flask in the shaking waterbath and shake at 250 rpm at 37 °C (or other desired temperature). Other temperature-controlled air-shaker can be used.
7. Monitor the OD_{600} and harvest cells at the density specified by different applications (see below).

3.2 Visualize B. subtilis Using Snapshot Microscopy

1. If cell membrane and DNA are going to be visualized, prepare the fluorescent dyes before the culture is ready: add 1 μl of 100× FM4-64 stock and 1 μl of 100× DAPI stock into 100 μl of growth medium (same medium used for growth, *see* **step 3** in Subheading 3.1). Protect from light.
2. While the cells are growing, prepare 2% agarose solution in growth media for making agarose pads: dissolve 0.6 g of molecular grade agarose in 30 ml of growth medium. Microwave until homogenous. Keep at 65 °C (*see* **Note 10**).
3. Prepare the agarose pads (Fig. 1) (*see* **Note 11**). Prepare two spacers by putting three layers of lab tape on a glass slide (*see*

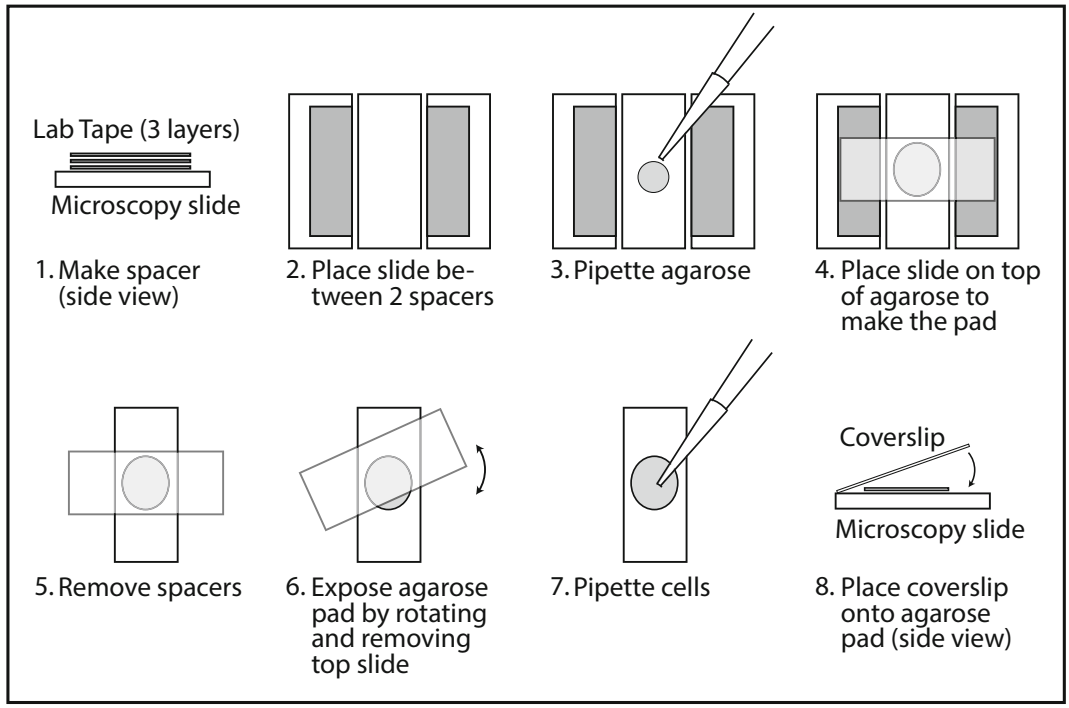

Fig. 1 Making an agarose pad for snapshot imaging. *Steps 1* and *8* are side views. *Steps 2–7* are top views

Note 12). Clean two microscopy slides per sample by wiping them with a kimwipe wetted with ethanol using a squirt bottle. Place one of the slides between the two spacers and add 100 µl of the 2% agarose solution to the center of the slide (*see* **Note 13**). Immediately after, gently drop a second slide over the agarose droplet. This will make a thin agarose pad "sandwiched" in between the two glass slides (Fig. 1) (*see* **Note 14**). Leave on the bench for 2–5 min to allow the agarose to solidify. Carefully remove the spacers from the agarose sandwich and remove one of the slides to expose the agarose pad (*see* **Note 15**).

4. Harvest cells when OD_{600} is between 0.2 and 0.4. Take 1 ml of culture and spin at 3300 × *g* for 30 s. Remove 900 µl of supernatant by pipetting or aspiration. Repeat centrifugation and remove supernatant completely using a P200 tip (*see* **Note 16**).
5. Resuspend the cell pellet using 10 µl of medium containing FM4-64 and DAPI from **step 1**. Pipette to mix and spot 1 µl of cells on an agarose pad prepared at **step 3**. Place a coverslip over the pad and visualize using the microscope (Fig. 2) (*see* **Note 17**).

3.3 Visualize B. subtilis Using Time-Lapse Microscopy

1. Grow the cells in CH or S750 minimal medium as in Subheading 3.1 to an OD_{600} of 0.2–0.4.
2. Prepare the stage top incubator for imaging. Carefully fill the water reservoir to maintain high humidity in the incubator (*see* **Note 18**). Turn on the stage top incubator and objective heater and allow them to reach and stabilize at the required temperature, which takes about 30 min. If other environmental chamber is used instead, set it up beforehand to let the temperature stabilize.

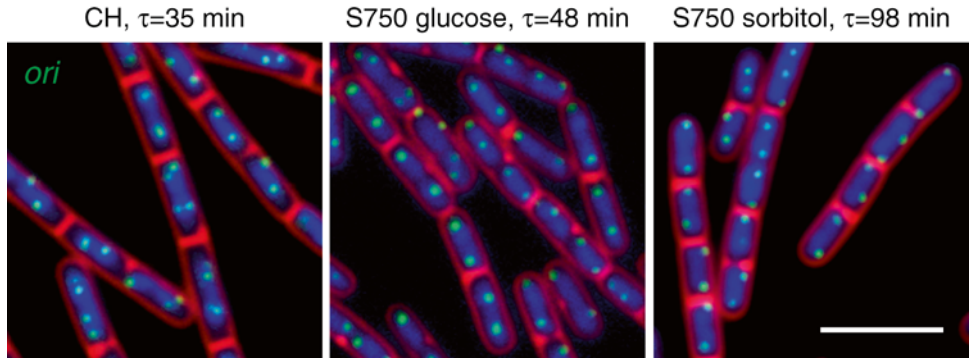

Fig. 2 Snapshot micrographs of cells growing in CH medium, or S750 minimal medium supplemented with glucose or sorbitol [11]. Membranes (*red*) were stained with FM4-64, nucleoids (*blue*) were stained with DAPI, origins (*green*) were labeled using *tet048*/TetR-CFP. τ, doubling time. Bar, 4 µm

3. Prepare 2% agarose solution in growth medium as in **step 2** in Subheading 3.2.
4. Pour 6 ml of the 2% agarose solution into a 60 × 15 mm petri dish and leave on the bench for 15–20 min (Fig. 3) (*see* **Note 19**).
5. Using a scalpel, cut 0.5 × 1.5 mm strips of agarose from the petri dish.
6. Take 1 ml of culture and spin at 3300 × *g* for 30 s. Remove 900 µl of supernatant by pipetting or aspiration. Resuspend the cell pellet using the remaining medium. Gently pipette up and down to mix (*see* **Note 20**).
7. Spot 1–2 µl of cells on a glass bottom dish, which will be used as a cover glass for the microscope. Lay a strip of agarose pad on top of cells using the scalpel (*see* **Note 21**).
8. Put the glass bottom dish containing cells and agarose pads in the stage top incubator. Close the lid of the incubator. Incubate for 15–30 min before imaging to stabilize the temperature in the pads and in the incubator (*see* **Note 22**).
9. To reduce image drift due to evaporation, chose a field of view that is not too close to the edge of the agarose strip. Optimize image acquisition to reduce photobleaching and phototoxicity (*see* **Note 23**). Take images at desired time intervals (Fig. 4).

3.4 Visualize B. subtilis During Sporulation

1. Grow the cells in CH medium as in Subheading 3.1.
2. While the cells are growing, prepare 100 ml of sporulation resuspension medium in a sterile bottle by combining 84 ml of

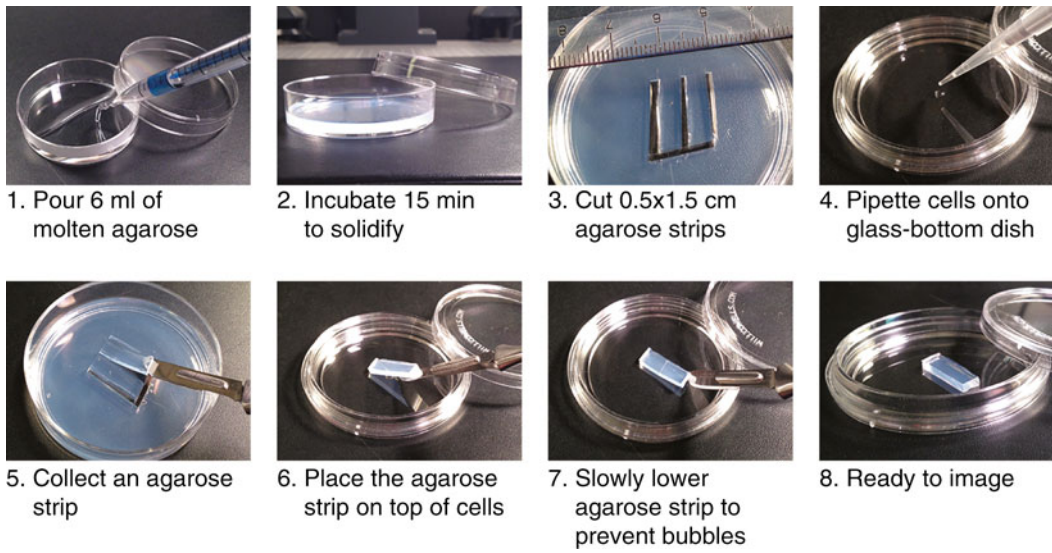

Fig. 3 Set up a slide for time-lapse microscopy using a glass bottom dish. During imaging, the agarose pad is fully exposed in the humidified temperature-controlled incubator

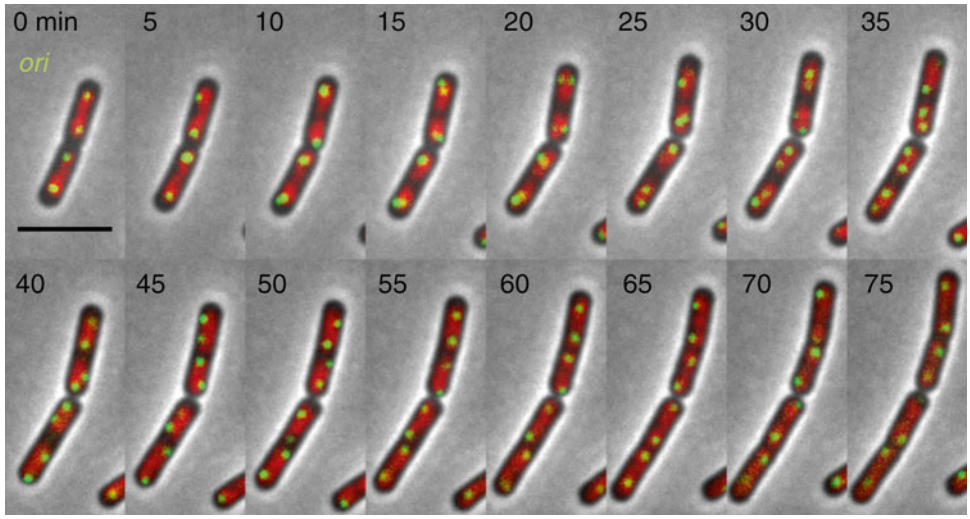

Fig. 4 A time-lapse progression (5-min intervals) of cells growing in S750 sorbitol minimal medium [11]. Nucleoids (*red*) were visualized using HbsU-GFP, origins (*green*) were labeled using *tet048*/TetR-CFP. Bar, 4 μm

ddH_2O, 100 μl of Solution A, 4 ml each of Solutions B, C, D, and E.

3. Prepare the fluorescent dyes in sporulation resuspension medium as in **step 1** in Subheading 3.2 (*see* **Notes 3**).
4. Prepare a 2% agarose solution in sporulation resuspension medium as in **step 2** in Subheading 3.2. Prepare agarose pad right before use as in **step 3** in Subheading 3.2.
5. When the culture reaches an OD_{600} of 0.5, transfer all the cells from the flask (about 20–25 ml) into a 50 ml conical tube by pouring. Save the empty flask for **step 7**.
6. Spin the tube in a bench-top centrifuge at 5000 × *g* for 5 min. Remove the supernatant by aspiration (*see* **Note 24**).
7. Add 20 ml of resuspension medium (from **step 2**) to the cell pellet. Pipette to resuspend and transfer the cells back to the original flask (from **step 5**). This is the time 0. Take a sample for microscopy if needed.
8. Put the flask back to the shaking waterbath and take samples at required time points to examine the progression of sporulation.
9. At each time point, take 200 μl of cells, spin at 3300 × *g* for 30 s, remove and discard the supernatant, and resuspend the cells in 10 μl of sporulation resuspension medium containing dye from **step 3**.
10. Prepare the slide as in **step 3** in Subheading 3.2 (Fig. 1) and visualize using a microscope (Fig. 5).

4 Notes

1. Plates can be stored at 4 °C for up to 3 months. Tetracycline plates should be protected from light.
2. Use deionized water (dH_2O) here, which contains trace amount of minerals.
3. FM4-64 and DAPI can be used at the same time. FM4-64 is not compatible with fluorescent fusions to mCherry. If membrane needs to be visualized together with an mCherry fusion, the blue membrane stain TMA-DPH can be used. In this case, to visualize the DNA, a GFP fusion to the nucleoid-associated protein HbsU (HbsU-GFP) [11] can be used instead of DAPI, which uses the same filter set as TMA-DPH. For sporulation, if a later time point is needed, TMA-DPH a preferred membrane dye because it is semi-permeable to cell membrane and the spore membrane can be visualized even after the spore is fully engulfed by the mother cell, while FM4-64 is impermeable to membrane and cannot stain the spore membrane once it is fully engulfed (Fig. 5).

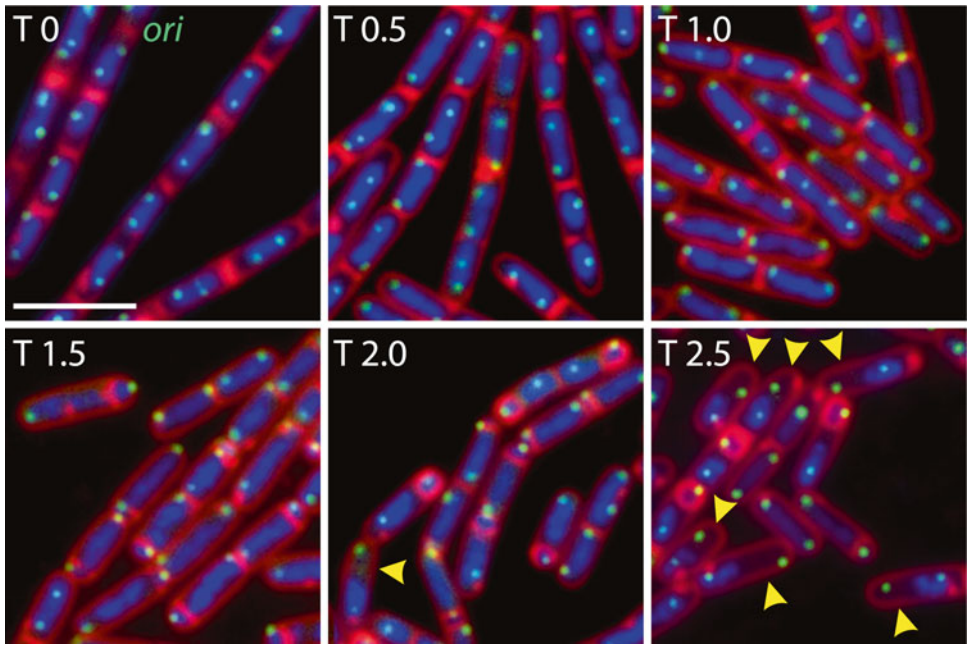

Fig. 5 Sporulation time course. Membranes (*red*) were stained with FM4-64, nucleoids (*blue*) were stained with DAPI, origins (*green*) were labeled using *tetO48*/TetR-CFP. Time (in hours) after the initiation of sporulation is indicated. Bar, 4 μm. *Yellow carets* indicate the fully engulfed spores, the membrane of which cannot be stained using FM4-64 (*see* **Note 3**)

4. Streak out strains freshly for every experiment.
5. Do not leave the plates at 4 °C. *B. subtilis* cells on the plates die at 4 °C.
6. CH medium has lower background fluorescence compared to LB medium.
7. *B. subtilis* tends to clump in the colony. Vortex gently for 5 s to make a homogenous inoculum.
8. This is to make sure that one of these two cultures will be in mid-exponential phase in the next morning.
9. To ensure adequate aeration, the volume of the medium used should not be more than 1/10th of the volume of the flask.
10. To prevent agarose from boiling over, heat the agarose solution in the microwave at 5 s pulses and gently swirl the bottle to mix thoroughly between pulses, until the agarose is fully melted. Use caution to prevent burns.
11. Prepare the agarose pads 2–5 min before harvesting the cells. Pads can be made 1–2 h in advance but need to be stored in a humid chamber to prevent them from drying off.
12. Spacers can be reused.
13. To prevent formation of bubbles, pipette the agarose solution using a pipette tip with its tip cut off.
14. Position the second slide over the agarose droplet and let it drop. This prevents the agarose pad from being slanted, which will produce uneven focal planes in the field of view while imaging.
15. To expose the agarose pad, carefully slide one of the microscopy slides over the other one. The agarose pad will remain adhered to one of the slides. Do not pull the slides apart because it can distort the agarose pad. Pads made this way are even in thickness. The slide that is removed can be reused.
16. *B. subtilis* grows in filaments. Higher speed of spinning could potentially shear the cells and affect protein localization. Cell pellet is loose so aspirate or pipette with care to prevent losing too many cells.
17. To prevent bubbles forming between the agarose pad and the coverslip, put the coverslip at an angle over the pad and gently lower it until it touches the agarose. To prevent depletion of oxygen from the cells, do not let cells sit in the agarose pad for longer than 10 min before imaging.
18. This is to prevent the agarose pad from drying out.
19. Do not over-dry the agarose pad.
20. We do not use dyes for time-lapse imaging because they may affect cell viability over time. If DNA needs to be visualized, a

fluorescent fusion to the nucleoid-associated protein HbsU, such as HbsU-GFP [11], can be used.

21. As with the coverslips, angle the agarose strip and slowly lower it onto the cells, to prevent bubbles. If the microscope stage allows multi-position acquisition, multiple strains can be visualized simultaneously on the same dish by putting cells and agarose strips in parallel, each for a different strain.
22. The agarose pad is fully exposed to oxygen to help growth of *B. subtilis* cells on the slide.
23. Optimize image acquisition by introducing Neutral Density (ND) filters in the light path and adjusting exposure times and time intervals. It is less damaging to the cell to introduce an ND filter and increase the exposure time than to only reduce the exposure time. This optimization is especially important when imaging *B. subtilis*. Phototoxicity causes the chromosome to expand and fill the entire cell compartment and it stalls cell growth and affects the dynamic behavior of many cell wall related proteins.
24. The cell pellet is not tight. Try to remove as much medium as possible without losing cells.

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