Methods for Studying Membrane-Associated Bacterial Cytoskeleton Proteins In Vivo by TIRF Microscopy

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Chapter 8

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Abstract

MreB proteins are actin homologs present in nonspherical bacteria. They assemble into membrane-associated discrete filamentous structures that exhibit different dynamic behaviors along the bacterial sidewalls. Total internal reflection fluorescence (TIRF) microscopy, a sensitive method for studying molecular events at cell surfaces with high contrast and temporal resolution, is a method of choice to characterize the localization and dynamics of cortical MreB assemblies in vivo. This chapter describes the methods for visualizing fluorescently tagged MreB proteins in live *Bacillus subtilis* cells. We detail how to (1) grow *B. subtilis* strains for reproducible TIRF observations, (2) immobilize cells on agarose pads and (3) in CellASIC® microfluidic plates, and (4) acquire TIRF images and time lapses.

Key words TIRF microscopy, Actin-like MreB proteins, Bacterial cytoskeleton, Agarose pad, CellASIC® microfluidic system, *Bacillus subtilis*

1 Introduction

MreB proteins are structural homologs of eukaryotic actin present in nonspherical bacteria and central to rod-shape determination. Over the last two decades, the use of fluorescent proteins and developments in imaging approaches have revealed that dynamic polymers of MreB associated with the cytoplasmic membrane localize along the cell cylinder, where they are believed to spatiotemporally coordinate enzymes involved in sidewall elongation. Among the fluorescent microscopy methods used to visualize the localization and dynamics of proteins in live cells, total internal reflection fluorescence (TIRF) microscopy stands as a method of choice for studying membrane-associated events in bacterial cells. In conventional wide-field microscopy setups, the incident light is parallel to the optical axis (i.e., the coverslip/sample surface normal) and thus travels across the entire sample, simultaneously exciting all fluorophores in the cell. Unfocused fluorescence from above and below

Helder Maiato (ed.), *Cytoskeleton Dynamics: Methods and Protocols*, Methods in Molecular Biology, vol. 2101, https://doi.org/10.1007/978-1-0716-0219-5_8,

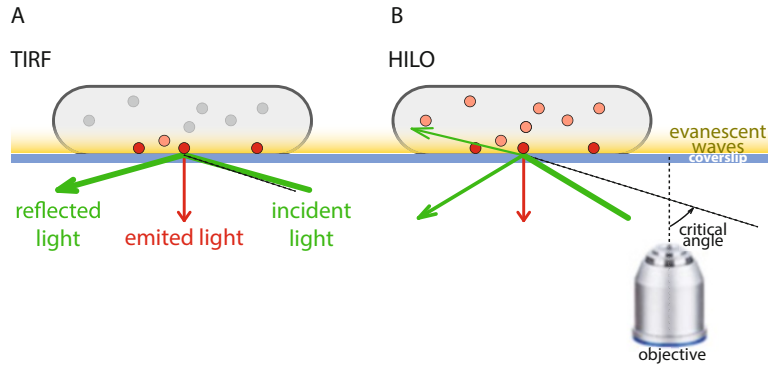

Fig. 1 Principle of TIRF (**A**) and HILO (**B**) illuminations. In a TIRF setup, the incident light hits the coverslip with a high angle relative to the optical axis (while in epifluorescence the light beam is in the axis of the objective; hence the angle is 0°). When the beam hits the coverslip/substrate interface with an angle higher than the critical angle (left panel, TIRF), all photons are reflected and the fluorophores (dots) are not directly excited. Only the evanescent waves generated at the interface and propagating in its close vicinity (yellow area) can excite the neighboring fluorescent particles (red dots). When the angle between the objective and the incident light is reduced below the critical angle (right panel, HILO), a fraction of the photons enters the substrate and a mixed illumination occurs ("dirty TIRF"). The proportion of reflecting photons decreases with the angle, to be minimum when the beam is in the axis of the objective (epifluorescence)

the focal plane is detected generating a high background scatter and thus a low signal-to-noise ratio (contrast). Two related techniques compatible with wide-field setups, TIRF and highly inclined laminated optical sheet (HILO), have been successfully applied to bacterial cells. In TIRF microscopy, the laser beam arrives on the sample with a high incident angle (relative to the optical axis) in which light is totally reflected (critical angle) (Fig. 1A). An evanescent wave is generated at the interface between the coverslip (glass) and the sample/water (aqueous medium), which have different refractive indices. The energy of the evanescent wave decays exponentially into the sample, selectively exciting the fluorophores at the proximity (100–200 nm) of the coverslip [1]. This enables a high contrast and, as a consequence, a high temporal resolution of molecular events at the cell surface. In HILO, the laser incident angle is a bit lower than the critical angle (subcritical angle) and a fraction of the photons penetrates the sample with a highly inclined angle, creating a mixed regime (Fig. 1B). HILO is therefore a middle ground between wide-field and TIRF illumination and can be useful for observations requiring a deeper penetration than TIRF microscopy, but it generates larger amounts of out-of-focus blur and leads to faster bleaching of the fluorophores [2]. When imaged by TIRF microscopy, *B. subtilis* MreB proteins appear as

spherical or elliptical assemblies close to the diffraction limit (light microscopy lateral resolution ~250–300 nm) that are evenly distributed along the bacterial cell cylinder [3, 4]. Growing cells contain subpopulations of both nonmobile (constrained) and mobile MreB assemblies, the latter exhibiting a variety of dynamic behaviors that require a high temporal resolution to be imaged: directed processive movement around the cell circumference, random diffusion, and mixed patterns of motions [4, 5].

In this chapter, we describe the methods to grow and immobilize *B. subtilis* cells expressing fluorescently labeled proteins for TIRF acquisitions. Although growing *B. subtilis* in a rich medium such as LB is easy, it is important to take into account a number of factors in order to achieve quality imaging experiments and good reproducibility, and to avoid generating artifacts. The bacterial cytoskeleton is very sensitive to the cell environment and growth conditions. The localization and dynamic properties of actin-like MreB proteins are quickly modified by stresses and are very sensitive to moderate changes (e.g., the speed of MreB assemblies exhibiting directed movement drops in a few seconds when cells are exposed to certain drugs or if the temperature is reduced) [3, 6, 7]. Thus, it is important to control carefully the growth and image acquisition conditions to minimize perturbations that may affect protein localization and/or dynamics.

Because bacteria do not directly adhere to glass and many, like *B. subtilis*, are motile, it is also important to immobilize them to allow quality microscopy acquisitions. Immobilization on agarose pads is minimally perturbative for bacterial cell physiology and therefore the main method used. Furthermore, it allows the growth of bacteria over extended periods under the microscope. Glass functionalization by adhesive molecules is a potential alternative to immobilize bacteria but does not reproducibly support growth over long periods and can deeply affect cell physiology. Cationic polymers such as poly-L-lysine, for example, have been shown to perturb the bacterial cell membrane and the proton motive force, which can affect the localization and/or dynamics of membrane-associated processes [7–9], and thus poly-L-lysine-coated slides are not recommended. Slide and agarose pad mounting can be advantageously replaced by a microfluidic chamber for long time-lapse experiments. In addition to immobilizing cells and supporting growth under constant controlled conditions, microfluidic systems allow the analysis of dynamic perturbations (e.g., change of growth medium, addition of drugs, etc.). Homemade microfluidic devices that allow growing bacterial cells in dead-ended channels (e.g., the "mother machines") or traps are ideal because they are customizable and cheap but require access to facilities and expertise. Here, we describe the use of the commercial CellASIC® ONIX Microfluidic System from Merck, which is a user-friendly and convenient alternative.

The methods described have been optimized for *B. subtilis* but should be generalizable, with some small adaptations, to TIRF imaging of other aerobic bacterial species.

2 Materials

2.1 Bacterial Cell Growth

Bacterial growth requires basic sterile microbiology vessels (test tubes, flasks, or microplates) in glass or plastic, with a cap or lid (to avoid contamination). The only restriction for aerobic bacteria such as *B. subtilis* [10] is, in addition to continuous strong agitation of the culture, to use a recipient large enough to allow a maximum aeration, i.e., the medium should not occupy more than 1/5th of the total volume. A variety of media with different formulations can be used depending on the bacterial species and specific application (transformation, protein expression, microscopy, etc.). Commercial LB medium (lysogeny broth, sometimes referred to as Luria broth or Luria-Bertani medium) is the most widely used rich medium and is routinely used for the study of the *B. subtilis* cytoskeleton [3–5]. However, cell suspensions in LB medium display background autofluorescence, which limits overall sensitivity and thus can be an issue for the observation of weak fluorescent signals. Non-autofluorescent media should be considered instead when needed. Cell growth is monitored by following the optical density of the culture at the single 600 nm wavelength ($OD_{600\ nm}$) using a spectrophotometer.

2.2 Agarose Solution for Pad Preparation

1. Ultrapure agarose (molecular grade).
2. LB (or alternative) medium or sterile pure water.
3. Microwave.
4. Standard microscopy glass slides 26 × 76 mm.
5. Coverslips 24 × 60 and 22 × 22 mm (thickness 170 μm, No. 1.5).
6. Lab tape or commercial frame.

2.3 Microfluidic Device

1. CellASIC® ONIX (Merck) Microfluidic System for live cell imaging.
2. Optically clear CellASIC® ONIX plates for bacterial cells (Ref. B04A).

2.4 TIRF Imaging

Use an inverted microscope equipped with an environmental chamber for temperature control (unless experiments are to be performed at room temperature), a laser, a motorized TIRF arm for incident laser angle control, a high-quantum-yield ultrasensitive camera (e.g., up to 95% quantum efficiency for back-illuminated EMCCD cameras, or >82% for sCMOS), and a 60× or 100× TIRF

oil-immersion objective with high numerical aperture (NA > 1.45). Immersion oil permits to use the full numerical aperture of the objective by removing reflection in the light path at the glass slide interface when refractive indexes of the immersion oil and the glass match. Refractive index depends on temperature (manufacturers usually provide its value at 23 °C) and should be adapted depending on specific acquisition conditions (e.g., 37 °C). Immersion oils must have extremely low or no fluorescence to avoid signal degradation due to undesired background. They can be purchased from most microscopy manufacturers (e.g., Immersol 518F, $n = 1.518$ @ 23 °C, Zeiss). The microscope stage must accept the 96-well plate format if microfluidic CellASIC® plates are to be used.

3 Methods

3.1 Growth of Bacillus subtilis Strains for TIRF Microscopy

1. On day 1, prepare a pre-culture by inoculating 2 mL of medium (e.g., LB or the medium to be used for the experiment, supplemented with antibiotics and/or with other supplements when needed) from a frozen glycerol stock of the strain of interest and incubate overnight. Alternatively, inoculate the pre-culture with a single colony from a freshly streaked plate (*see* **Note 1**). Adjust the duration of the pre-culture and the temperature of incubation in order to minimize entering into stationary phase. Typically, start the pre-culture as late as possible, grow it at 25–30 °C with strong aeration, and dilute it the next morning.
2. On the day of the experiment, measure the optical density ($OD_{600\ nm}$; *see* **Note 2**) of the pre-culture and make an appropriate dilution into fresh medium to a theoretical OD_{600} of 0.005 (*see* **Note 3**). If comparing several strains, it is important to ensure an identical starting cell density so that all the strains will be observed at an identical OD_{600} after an identical number of generations. Do not add antibiotics to the culture since growth in the presence of antibiotics can affect growth rate and/or shape. If maintaining the selective pressure is absolutely required, include a control strain containing the same resistance marker.
3. Grow the cultures at constant temperature (e.g., 30 or 37 °C) and maximum agitation (no less than 200 rpm) for optimum aeration [10], and monitor bacterial growth by following the OD_{600}.
4. When the desired cell density is reached, add 1–2 μL of the bacterial culture on a pre-warmed 2% agarose pad, prepared as described below. To increase cell density on the agarose pad (especially if working at low cell density), the culture can be concentrated by gentle centrifugation (e.g., 1–3 min

at 1000 × *g*). If taking several samples throughout growth, be careful to minimize perturbations (e.g., do not leave the cultures standing on your bench for minutes while taking a sample; cells will detect both the aerobic and temperature downshift and reduce their growth accordingly).

3.2 Agarose Pad Preparation and Slide Mounting

1. Prepare a solution of 2% agarose: typically, microwave-melt 100 mg of ultrapure agarose in 5 mL of LB (or the growth medium used for the experiment or water; *see* **Note 4**) in a 50 mL conical tube. It is recommended to prepare the agarose solution daily (*see* **Note 5**).
2. To obtain agarose pads of the desired thickness, create spacers by sticking together two layers of lab tape and placing two of these on a microscope slide as shown in Fig. 2A (*see* **Note 6**).
3. Pipet approximately 250 μL of the melted 2% agarose solution between the two spacers, immediately cover the drop with a 24 × 60 mm coverslip (Fig. 2A, B), and place a weight on top to pressure the solution and create a uniformly flat surface. An aluminum-heating block makes a very good job as it is heavy and efficiently dissipates the heat, speeding up the agarose solidification process.

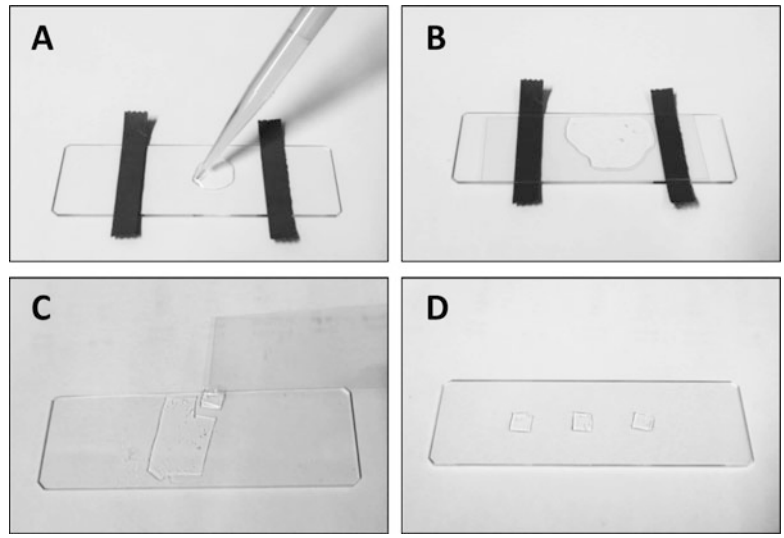

Fig. 2 Agarose pad preparation. (**A**) Stick two pieces of double-layered lab tape on a microscope slide to obtain spacers of the desired thickness. Pipet approximately 250 μL of the melted 2% agarose solution between the two spacers. (**B**) Immediately cover the drop with a 24 × 60 mm coverslip. Cover with a weight (not shown) and allow to set for a few minutes. (**C**) Use the edge of a clean coverslip to cut ~5 × 5 mm mini pads. (**D**) Example of three mini pads placed on the same slide

4. After a few minutes, carefully remove the weight, the coverslip, and the spacers, and use the edge of a clean coverslip to cut ~5 × 5 mm mini pads (Fig. 2C). Carefully lift these mini pads with an edge of the coverslip, and place them on a clean microscope slide. Several mini pads can be placed on the same slide (Fig. 2D) (*see* **Note 7**).
5. Add 1–2 µL of the bacterial culture at the center of the mini pad (ideally pre-warmed), and allow the pad to adsorb the suspension before placing a 22 × 22 mm coverslip on top. Gently press the coverslip to allow complete adherence to the pad. If multiple pads have been disposed on the glass slide, use a large 24 × 60 mm coverslip.

3.3 Microfluidic Setup (CellASIC® ONIX)

The CellASIC® device and plates should be used according to the manufacturer's recommendations. Here, we indicate specific tips based on our own experience for the preparation, loading, and mounting of the plates. We describe the simple case where bacteria are continuously perfused with a single growth medium, but changes of media can be achieved by using the extra wells (#2–5; Fig. 3).

1. Plate preparation: Completely pipet out the PBS buffer from the wells that will contain the cells (#9) and the medium for perfusion (#1) and for loading (#6), and wash once with fresh medium. Also, empty half of the waste (#8) to prevent overflow (Fig. 3). Then, fill in wells #1 and #6 with 1–200 µL of medium and #9 with 1–200 µL of bacterial cell culture.
2. Turn on the CellASIC® device, and seal the plate according to the manufacturer guidelines. Position the plate on the stage so

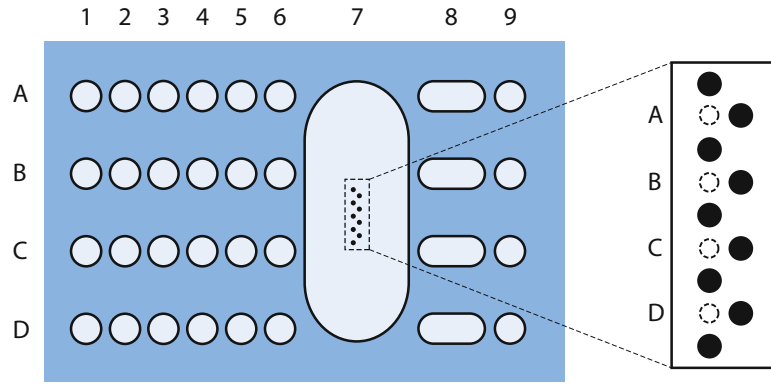

Fig. 3 CellASIC® ONIX B04A microfluidic plate. Schematics of the plate. The plate is organized in four rows (*A–D*) allowing four independent experiments, and nine columns corresponding to: (*1–5*), wells for media/solutions; (*6*), wells for medium for cell loading; (*7*), area containing the four culture micro-chambers (*A–D*); (*8*), trashes, and (*9*), loading wells for the cells

that the growth chambers are aligned with the objective (Fig. 3; *see* **Note 8**). The chambers should be visible in the field of view.

3. Load bacteria into the chamber using a 4 psi pressure (pressure determines the flow rate of medium) for 6 s. *B. subtilis* is typically trapped in the fourth area of the micro-chamber (this depends on cell diameter and can vary with species; *see* **Note 9**). Finally, apply a continuous (5 psi) flow of fresh medium (from well #1) to allow efficient bacterial growth. We recommend to check after a few minutes that the cells are indeed growing and to control frequently the absence of communication errors between the CellASIC® controller and the plate, which lead to a flow arrest.

3.4 TIRF Imaging

1. Put a small drop of microscopy oil on top of the coverslip (or on the objective), and place the slide or microfluidic plate on the temperature-controlled microscope stage. When possible, use white light illumination (bright-field, phase contrast, DIC, etc.) rather than fluorescence to find the focal plane containing the cells to prevent useless bleaching of the fluorophores.
2. Set up the TIRF settings so that the laser power is minimal and the laser/objective angle maximal, ensuring that there is no direct penetration of photons in the sample. From this starting point, carefully reduce the laser angle while adjusting the focus until the fluorescence appears. Stop reducing the laser angle as soon as the fluorescence is just visible enough for quality acquisition (*see* **Note 10**). If the slide has been correctly mounted, the laser angle and the focus can be kept unchanged over the entire surface of the slide if the microscope is equipped with a device that automatically maintains the focus (e.g., Nikon "PFS," Zeiss "Definite Focus", etc.). Such autofocus systems are extremely useful when using this type of mounting since the thickness of the agarose pad will gradually change due to water evaporation, directly increasing the objective/sample distance.
3. First, use continuous acquisition while varying the exposure time to get a sense of the dynamics of the particles. Then, adapt the acquisition parameters: very fast dynamics requires a high sampling rate, while low-intensity signals require longer exposure times. High sampling rate and long exposures both decrease the "photon budget" of the fluorophores.
4. Change the field of view to find new cells, and image particle dynamics using the optimized frame rate and illumination. Typically, for *B. subtilis* fluorescently tagged MreB proteins, a rate of 0.5–2 frames/s is used when imaging exponentially growing cells at 37 °C (when fluorescent particles travel at 50–100 nm/s) [3, 6].

4 Notes

1. Starting a culture directly from a frozen stock (without pre-culture) is not appropriate because there is no control of the number of (living) cells inoculated, and thus of the number of generations that will occur during the experiment. It is possible to streak the strain on a fresh plate instead, but this is less convenient to control the cell density when starting the culture (*see* **Note 3**). Also, do not use old streaks (>24 h plate) because the cells will enter a variety of stationary-phase processes including sporulation and lysis, generating a broad spectrum of cellular states.
2. Keep in mind that turbidity (optical density) (a) is a value related to the dry mass of the population (not to the number of cells or the cfu (colony-forming units)), (b) can vary between spectrophotometers depending on their calibration (thus an "$OD_{600} = 0.5$" gives little information on the exact growth phase of the bacterial culture without the corresponding growth curve), and (c) is proportional to cell mass in the range of linearity of the material, which must be predetermined. A growth curve of your reference strain must be done first using the spectrophotometer that will be used for the experiments. This will reveal the OD_{600} where growth phase change(s) occur(s), which should be constant for a given growth condition (temperature, medium, aeration) [11].
3. Start cultures at a low cell density to ensure that a sufficient number of generations in exponential growth will occur.
4. Ideally, the solvent used to melt the agarose should be the same medium used for growing the cells (e.g., LB), if it supports microwaving. If not, an agarose/water pad can be prepared and the water later exchanged with the medium by soaking the pad for half an hour in a petri dish filled with the medium. In some cases, cells are also observed directly on a water/agarose pad, but keep in mind that the cells will face a rapid dilution of their growth medium when added to the water pad, which can cause stress to the cells and consequently affect localization of the proteins of interest.
5. Melted agarose in medium should be prepared fresh of the day but can be kept several days at 55 °C when prepared in water. Agarose pads should not be kept more than a few hours at room temperature to avoid desiccation (in all cases) and contamination when prepared with medium.
6. Commercial frames (e.g., "gene frame" from Thermo Fisher, Ref. AB0577) can be used instead of lab tape spacers. However, the custom-made tape spacers are infinitely cheaper and allow preparing pads than can be cut into smaller pieces to image

multiple samples on a single slide (*see* Fig. 2D), minimizing cost and manipulations. The main benefit of commercial frames is that by sticking to both the glass and the coverslip, they limit dehydration of the agarose pad.

7. The mini pads should be used right away to limit the evaporation process. The uncut agarose pad covered with the coverslip can be kept for a couple hours prior to use. Place it in a petri dish containing extra moisture, especially if it is to be kept in a warm room or in the oven so that it is at the same temperature than the bacterial culture to be imaged.
8. To position the objective on top of the four micro-chambers (dotted circles on the zoomed-in area in Fig. 3), which are too small to be seen with the naked eye, use as landmark the holes (black circles) that are surrounding them.
9. The micro-chambers contain six successive areas with a step-like, decreasing height of the microfabricated elastic silicone ceiling. The cells enter through the most spacious area and are pushed by the flow into the direction of the low-ceiling zones until they become immobilized, physically trapped in the area with height similar to their diameter. The B04A plate has heights of 4.0, 3.0, 2.0, 1.1, 0.9, and 0.7 μm. *B. subtilis* is usually found on areas 4 or 5.
10. Signal intensity will progressively increase as the angle is reduced, as some (and more and more) photons in addition to the evanescent waves will penetrate the sample. This intermediate state in which the cell is partially illuminated by photons with high incident angles is HILO, also referred to as "dirty TIRF" (Fig. 1). The transition from TIRF to HILO illumination is gradual when decreasing the laser angle and thus difficult to perceive. The goal is to optimize as much as possible the TIRF angle in order to minimize the penetration depth as well as bleaching and background noise.

Acknowledgment

This work was supported by a Consolidator grant from the European Research Council (ERC COG) under the European Union's Horizon 2020 research and innovation program (grant agreement No. 772178) to R.C.-L.

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