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Single-Molecule Imaging of *Escherichia coli* Transmembrane Proteins

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Abstract

Single-molecule imaging in living cells can provide unique information about biological processes. Bacteria offer some particular challenges for single-molecule imaging due to their small size, only slightly larger than the diffraction limit of visible light. Here, we describe how reliable and reproducible single-molecule data can be obtained for a transmembrane protein in the Gram-negative bacterium *Escherichia coli* by using live-cell fluorescence microscopy. Fluorescent labeling of a protein by genetic fusion, cell culturing, sample preparation, imaging, and data analysis are discussed.

Key words

Single-molecule tracking
Bacteria
Escherichia coli
Transmembrane protein
Diffusion
Fluorescence microscopy
Sample preparation

1. Introduction

Fluorescence microscopy is a powerful technique that enables scientists to probe macromolecular organization, localization, and dynamics in living cells. However, the maximum resolution achieved in standard fluorescence microscopy is intrinsically limited by the diffraction of light. This limitation is a serious problem for imaging bacteria, since the maximal resolution (~250 nm) is comparable to the size of the entire cell (typically ~1–2 μm). As a result, the structures and dynamics of key bacterial machineries, often smaller that the diffraction limit, are hard to be probed in vivo. In this chapter we describe a protocol for fluorescence labeling and imaging of transmembrane proteins that allows detection of single molecules within live *E. coli* cells and allows locating them with a better accuracy than the diffraction

limit. To achieve this, we genetically fuse a fluorescent reporter to the amino-terminus or carboxy-terminus of the protein of interest. Then we clone the labeled protein into a low expression plasmid that produces ~10–100 fluorescently labeled protein molecules per cell. These cells are grown in a shaking flask and imaged on a temperature-controlled microscope stage. Using wide-field fluorescence microscopy, individual fluorescent protein molecules within bacterial cells are visualized and their mobility is tracked using single-particle tracking software.

2. Materials

1. PCR Master Mix: mix 1–2 µl of template DNA (1–10 ng for plasmid DNA, up to 250 ng of genomic DNA), 2.5 µl (10 pmol) forward primer, 2.5 µl (10 pmol) reverse primer, 1.4 µl of a 5 mM dNTP solution, 5 µl of 10× concentrated polymerase buffer (provided with the enzyme), 1 µl (30 U/µl) high fidelity DNA polymerase, and add nuclease-free water up to a volume of 50 µl.

2. Gibson Master Mix: 50 µl Taq ligase (40 U/µl, New England Biolabs), 100 µl isothermal buffer (5× concentrated, NEB), 2 µl T5 exonuclease (1 U/µl, NEB), 6.25 µl Phusion polymerase (2 U/µl, NEB), 216.75 µl nuclease-free water. Store aliquots of 15 µl at –20 °C.

3. DNA Ligation Mix: 2 µl of 10× concentrated DNA ligase buffer (provided with the enzyme), 1 µl T4 ligase, 40 fmol of vector DNA (100 ng for a typical vector of 4 kb), and 60 fmol of insert DNA (37.5 ng of a typical insert of 1 kb) both restricted with the appropriate restriction enzymes; make the volume up to 20 µl with nuclease-free water.

4. YT Medium: add 16 g Tryptone, 10 g Yeast Extract, 5 g NaCl in ~900 ml of distilled water, Adjust the pH to 7.0 with NaOH, make the volume up to 1 l with distilled water, sterilize the solution by autoclaving.

5. M9 Medium: dissolve 6 g Na_2HPO_4, 3 g KH_2PO_4, 1 g NH_4Cl, 0.5 g NaCl in 900 ml deionized water and autoclave the solution. Subsequently add from filter-sterilized stock solutions: 2 ml of 1 M $MgSO_4$, 0.1 ml of 1 M $CaCl_2$, 20 ml of 20% glucose, 10 ml of 10% casamino acids, 10 ml of 1% (w/v) thiamine. Make the volume up to 1 l with sterile water.

6. Agarose: very pure low melting agarose.

7. VALAP wax: 10 g Paraffin, 10 g Lanolin, 10 g Vaseline, slowly heated to 60 °C while gently stirring.

3. Methods

3.1. Plasmid Construction and Cloning

1. Amplify the DNA sequence encoding the protein of interest from the chromosome of the *E. coli* strain of interest using Polymerase chain reaction (PCR). Protocols for genomic DNA isolation, primer design, and PCR reactions are described elsewhere [1].

2. In order to fluorescently label the protein of interest, amplify the DNA sequence of a fluorescent

protein (*see* **Note 1**) and fuse it to the N-terminal or C-terminal end of the protein of interest by cloning the two genes in one open reading frame, using for example Gibson isothermal assembly [2]. It is important to verify that folding, localization, and activity of the protein of interest are not affected by fusion to a fluorescent protein. Appropriate control experiments should be performed in order to check the activity of the fusion protein. Parameters that can be varied in order to reduce the influence of the fluorescent protein on the target protein's folding and function include the place where the fluorescent protein is fused (N-terminus, C-terminus, or sandwiched in a loop) and the introduction of short, polar, flexible linkers between the fluorescent protein and the target protein (*see* **Note 2**).

3. Clone the fused fragments into a low or medium copy number plasmid (*see* **Note 3**) that allows tight regulation of protein expression, for example by Gibson assembly [2] or by restriction and ligation [1]. If the correct functioning of the protein of interest strictly depends on the correct expression level, one could consider replacing the endogenous gene in the *E. coli* genome by a fluorescently tagged version of the same gene, for example by homologous recombination [3] or by CRISPR-Cas9-based technology [4].

4. Verify the genes on the plasmid by sequencing.

5. Transform the plasmid that contains the fusion fragments into *E. coli* cells of interest by electroporation or heat-shock. Then plate the transformants on YT agar plates supplemented with the appropriate antibiotics, for example ampicillin (100 μg/ml) for pBAD24 or chloramphenicol (34 μg/ml) for pBAD33.

3.2. Cell Culture and Sample Preparation

1. Pick one *E. coli* colony from the YT agar plate and inoculate the cells in 2–5 ml of fresh YT medium containing the appropriate antibiotics. Incubate in a shaker at 37 °C, long enough to reach an OD_{600} (optical density at 600 nm) that exceeds 1.0. We typically grow the pre-culture over-night.

2. Dilute the culture 100× in 5 ml fresh YT medium with appropriate antibiotics and incubate in a shaker at 37 °C.

3. Turn on the fluorescence microscope and set the objective lens heater to the desired imaging temperature. We use a stage-top incubator system (Tokai Hit, INU-ZILCS-F1) for equilibrating the apochromatic 100× 1.49 NA TIRF oil-immersion objective to 23 °C. Leave the microscope at this setting for 90–120 min in order for temperature equilibration to be complete (*see* **Note 4**).

4. The cells are ready for imaging when the OD_{600} equals 0.3–0.4 (at 37 °C, with an initial OD_{600} of 0.02, this will take about 90 min) (*see* **Note 5**). Centrifuge the culture at 4000 rpm (1789 *G) for 2 min in a benchtop microcentrifuge in order to obtain the cell pellet. In the meanwhile, start preparing the agarose gel pad (Subheading 3.3).
AQ1

5. Discard the supernatant and add 5 ml fresh minimal medium M9 and resuspend the pellet gently (*see* **Note 6**).

6. Cells resuspended in 5 ml M9 medium can be directly used for short-term time-lapse imaging. For long-term time-lapse imaging, dilute the resuspended cells 10–100-fold in fresh M9 medium (*see* **Note 7**).

3.3. Preparation of Agarose Solution

1. Weigh approximately 75 mg agarose into a 5 ml tube.

2. Add appropriate volume (approximately 5 ml) of M9 minimal medium without antibiotics to make a 1.5% agarose solution.

3. Heat the agarose solution for 45–60 s in a microwave oven to dissolve the agarose. Shake the tube to ensure that the solution is completely clear and homogenous. The gel pad can be poured at this point (Subheading 3.4) or the agarose solution can be kept at 50 °C for several hours for later use.

3.4. Preparation of a Gel Pad on the Microscope Slide

1. About 60 min before imaging, clean the microscope slides and cover slip by blowing with compressed air. Then clean them with a plasma-cleaner (*see* **Note 8**). Each sample requires two slides and one cover slip.

2. Prepare two spacer slides by putting two layers of labeling tape on each of two microscope slides (Fig. 1). The microscope slides should have the same thickness as the ones used for imaging (*see* **Note 9**).

3. Clean the lab table with 70% alcohol and prepare the sample under a lit burner to avoid contamination of the slides. Alternatively, one could work in a laminar flow cabinet.

4. Place a clean microscope slide between the two spacer slides as shown in Fig. 1.

5. Apply 400 μl of the warm agarose solution (**step 3** in Subheading 3.3) to the center of the clean slide.

6. Rapidly top the agarose solution with a second clean slide as shown in Fig. 1.

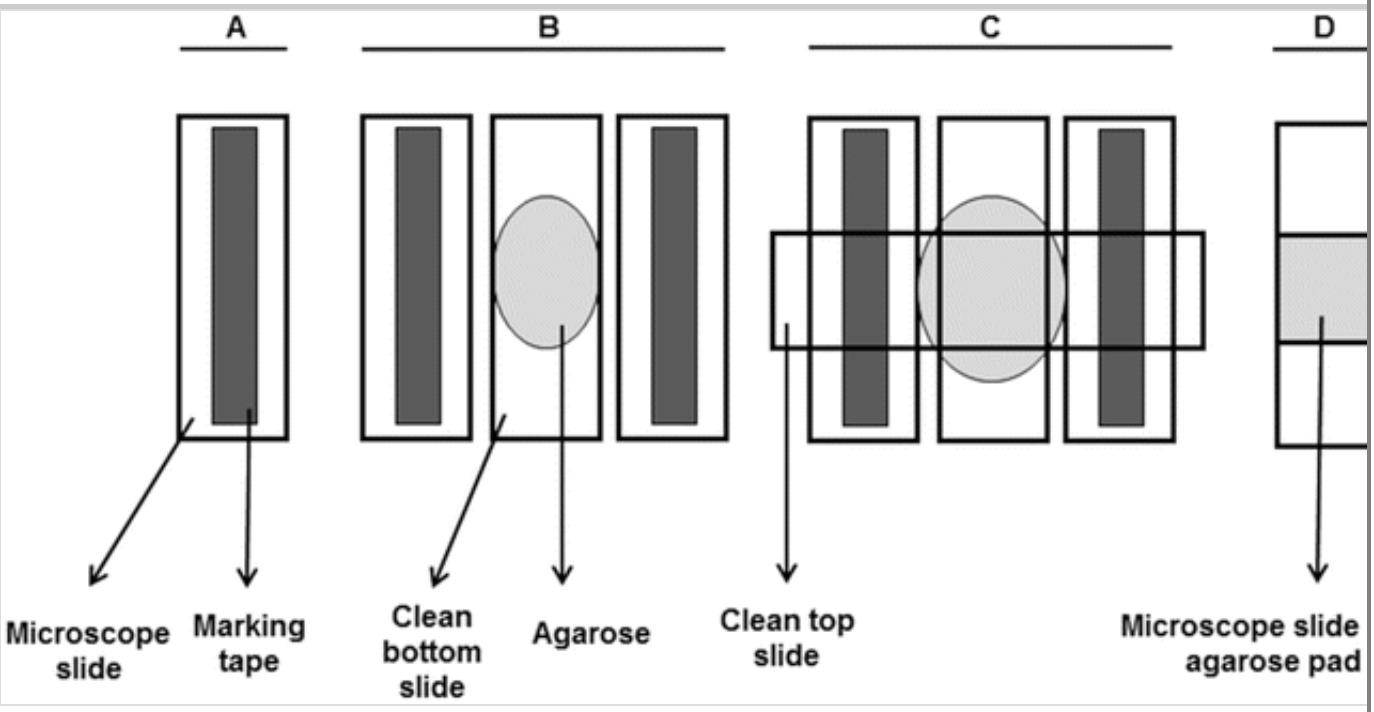
7. Allow the agarose solution to solidify at room temperature for 1 min. Then cut excess agarose around the top slide with a clean razor blade.

8. Carefully slide off the second glass slide from the top of the gel pad, and remove excess agarose gel at the sides of the bottom slide with a clean razor blade. Add 8 μl of cell culture suspended in M9 medium (from Subheading 3.2) to the top of the gel pad. Wait for ~20–30 s for the culture to be absorbed by the gel pad. It is important not to wait too long, such that the gel pad dries out, but long enough for cells to properly adhere to the gel pad. The ideal waiting time may vary with (room) temperature and humidity. Once the cell suspension is absorbed by the agarose gel, place a clean cover slip on top of the pad (*see* **Note 10**).

9. Seal the sample chamber with molten VALAP wax around the edges of the cover slip (*see* **Note 11**). sample can now be used for imaging on the microscope (*see* **Note 12**).

Fig. 1

Sample preparation for microscopy (**a**) Spacer slide with double-layered marking tape. (**b**) two sp slides flanking a clean bottom slide with a 400 μl of agarose dissolved in M9 medium. (**c**) A clea slide is added to level off agarose, (**d**) Finished slide with a thin square-shaped agarose pad



3.5. Time-Lapse Imaging

1. Mount the sample on top of the microscope objective equilibrated at the desired measuring temperature for at least 90 min (*see* **Note 4**).

2. Let the sample be on top of the objective for ~15 min (this will equilibrate the cells to the exact measuring temperature, *see* **Note 13**). In practice, we use this time to find regions of interest and modify imaging scripts and file names as necessary for an experiment.

3. Find cells on the microscope using bright-field transillumination and position them in the center of the imaging region and bring them into focus (*see* **Note 14**). Best results are obtained when the bottom half of the cell is in focus (*see* **Note 15**). More than one cell can be imaged in each image acquisition time window. For time-lapse imaging over several generations, ensure that imaged cells are initially separated from other cells by at least a few hundred micrometer so that other colonies will not enter the imaging region during growth.

4. Turn on the excitation laser with closed shutter, to avoid bleaching prior to acquisition. A laser intensity of ~200 W/cm^2 is required for imaging single molecules that diffuse in the membrane of a living bacterial cell (*see* **Note 16**).

5. Open the shutter and immediately record a continuous series of images until all fluorescent molecules have bleached. We typically record 200–300 images per region of interest. A sensitive camera is required to image single, diffusing fluorescent molecules (*see* **Note 17**).

6. Repeat **steps 3–5** until sufficient data have been recorded (*see* **Note 18**).

7. Images are analyzed to find the positions of individual molecules in each image and link the positions into trajectories, from which diffusion coefficients can be extracted. We use custom-written routines in MATLAB (MathWorks) described elsewhere [5, 6].

4. Notes

1. Our microscope has green and red detection channels. Out of the available green fluorescent proteins, we find that it is best to use eGFP for cytoplasmic labeling of *E. coli* membrane proteins due to its high photostability, high brightness, low blinking rate and fast maturation. We find that it is best to use sfGFP for periplasmic labeling of *E. coli* membrane proteins because of its robust folding and fluorescing property in the more oxidizing periplasmic environment [7]. Out of the red fluorescent proteins that we tried, mCherry gave the best results.

2. In our recent work, we chose to label the cytoplasmic N-terminal end of the following transmembrane proteins of *E. coli* strain MC4100 by fusion to green fluorescent protein (eGFP): YedZ, CybB, GlpT, CstA and WALP-KcsA [8]. For the transmembrane protein MscL we chose the cytoplasmic C-terminal end for eGFP fusion, because the N-terminus is located in the periplasm where eGFP does not mature. In the case of the transmembrane protein MscS, we chose the periplasmic N-terminal end for fusing to super folder green fluorescent protein (sfGFP) as the C-terminal end is involved heptamerization. The N-terminal sfGFP was translocated co-translationally across the cytoplasmic membrane via a signal sequence derived from the protein DsbA [8].

3. We typically use an arabinose inducible plasmid, pBAD24 or pBAD33, because of their moderate copy number and tight regulation of protein expression in bacterial cells [9].

4. When measuring diffusion of membrane proteins, it is extremely important that the temperature of the microscope is stable when starting the experiment. Diffusion depends strongly on temperature, and also the composition of the bacterial membrane alters upon temperature changes. Even small changes in temperature during data acquisition can lead to very complex heterogeneous data.

5. We usually choose midlog phase cells for imaging because at this phase *E. coli* cells are generally healthy and metabolically homogeneous, and produce most of the intracellular proteins. However, the experiment can be performed at other growth phases if required for studying particular cellular processes.

6. We use M9 minimal medium for imaging due to its reduced auto-fluorescent property which reduces background fluorescence signal during image acquisition. An even stronger reduction of auto-fluorescence can be obtained by growing the cells in minimal medium (**step 2**) at the cost of slower growth.

7. Low cell densities are important for extended time-lapse imaging. Due to exponential growth of cells, high initial cell concentrations will result in micro-colonies rapidly growing on top of each other, and it can significantly deplete oxygen in the gel pad after prolonged growth, reducing fluorescent-protein maturation and affecting cell growth.

8. We clean the microscope slide and cover slip using plasma cleaner to reduce background noise emerging from the glass surface during image acquisition. Glass slides often contain small fluorescent organic compounds that, when not removed, can easily be mistaken for fluorescent proteins during data acquisition. We use a Harrick Plasma cleaner by setting the RF level high for 15 min. We use 76 × 26 mm microscope slides from Menzel-Gläser with the thickness of about 1 mm, and 22 × 22 mm cover slips from Menzel-Gläser with the thickness of 0.16–0.19 mm.

9. The strips of tape act as spacers, they provide an easy means to achieve agarose pads with homogeneous and reproducible thickness. More layers of tape will lead to thicker pads. The TimeMed tape that we use has a thickness of about 0.13 mm.

10. We use agarose-pads for immobilizing bacterial cells because they provide a suitable environment for the cells to adhere gently on their surface with less physical pressure. On agarose pads, nearly all cells are lying horizontally, which is not the case in other immobilization methods that we tried.

11. We seal the microscope slides with VALAP to prevent the sample from drying.

12. We perform sample preparation at room temperature. The preparation, i.e., making the agarose pad, applying the cells and sealing the sample chamber with VALAP takes approximately 15 min.

13. We incubate the sample on the microscope for 15–30 min to allow the cells to adjust to the imaging temperature (23 ± 1 °C). This is important because we initially grow cells at 37 °C in a shaking flask, then prepare the sample at room temperature, and later image them at 23 °C. Drastic temperature shift will cause significant changes in cellular functions and membrane lipid composition. Even small shifts in temperature during acquisition, in the order of 0.1 °C lead to significant drift of the diffusion coefficient. Therefore, allow sufficient time for the cells to adapt to required measuring temperature. We strictly follow the sample preparation and incubation timing (i.e. from the point where the cells are resuspended in M9 medium to imaging) to get reliable and reproducible data. The entire procedure is summarized in Fig. 2.

14. We typically use a motorized microscope stage controlled by a joystick (Applied Scientific Instrumentation, MS-2000). We then use the motorized focus system to bring the cells into right focus.

15. For the high-magnification objectives that are required for single-molecule tracking in bacteria, focal depth is approximately 500 nm, and *E. coli* cells are approximately 1 μm in diameter. Focusing to the middle of the cell thus has the disadvantage that the membranes on the top and bottom of the cell will not be in focus.

16. To image eGFP or sfGFP, we use a 491 nm diode-pumped solid-state laser (Cobolt Calypso 50™ 491 nm DPSS), in combination with a dichroic mirror (Semrock, 488/561 nm lasers Brightline® dual-edge laser-flat, Di01-R488/561-25×36) and an emission filter (Semrock, 525/50 Brightline®

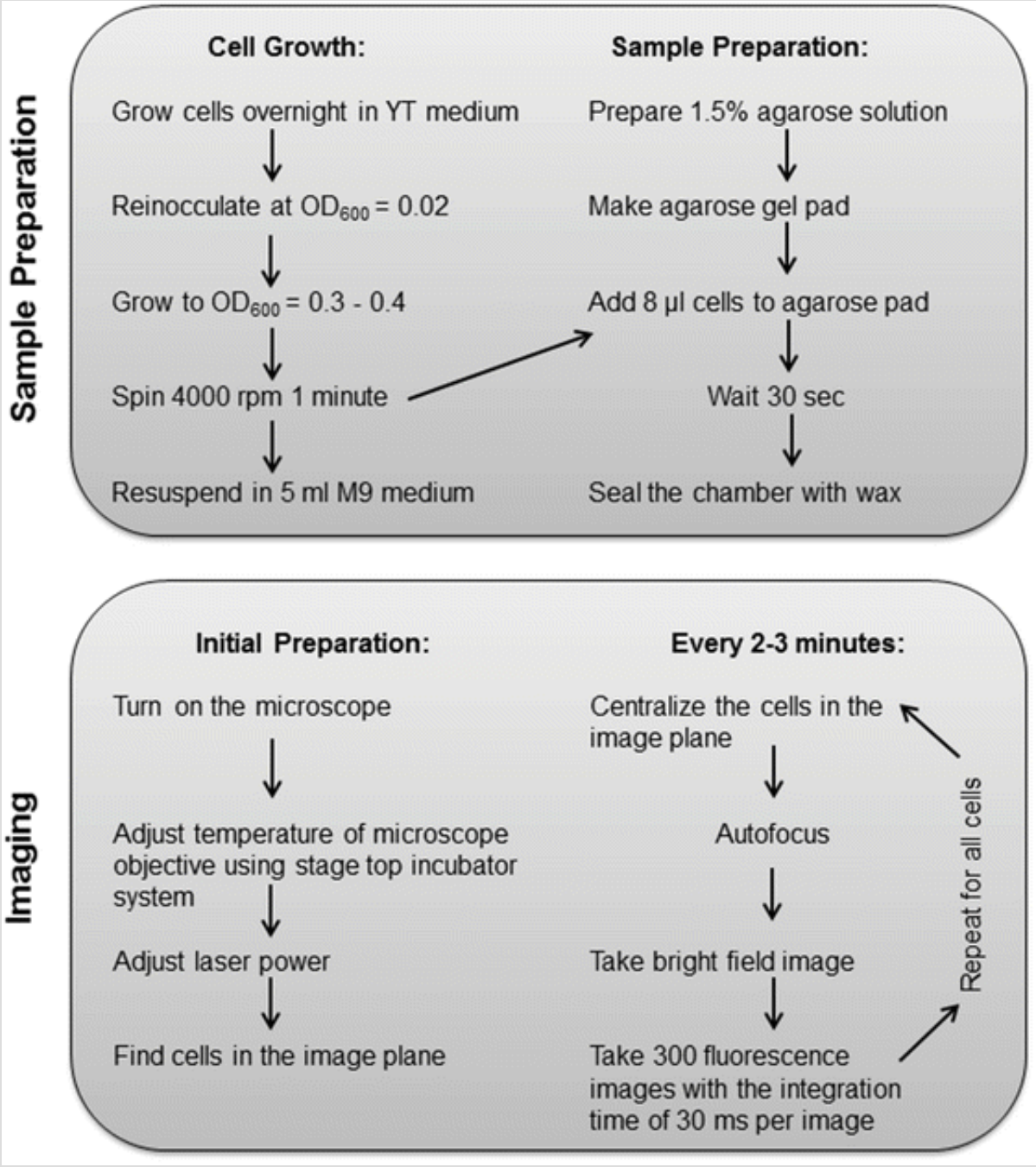
single-band band pass filter, FF03-525/50-25).

17. We use an EMCCD camera (Andor iXon3, type 897) for acquiring fluorescence images continuously with an integration time of 32 ms per image. We use a total magnification of 200×, corresponding to 80 nm by 80 nm in the image plane per pixel.

18. We acquire images for approximately 40–60 min. Longer imaging will lead to data collection from aging cells which are generally smaller in size and less fluorescent, which might be due to nutrient depletion or metabolic changes.

Fig. 2

Workflow of sample preparation and imaging of fluorescently labeled transmembrane proteins in *E. coli*



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