

Single Molecule Localization Microscopy (SMLM) imaging of bacteria: A sample preparation guide

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Method Article

Keywords: Super-resolution microscopy, Single-Molecule Localization Microscopy, Direct Stochastic Optical Reconstruction Microscopy, Bacteria, E.coli, Outer membrane proteins, Spatial organization,

Posted Date: October 1st, 2020

DOI: <https://doi.org/10.21203/rs.3.pex-1094/v1>

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Abstract

In recent years Super-resolution microscopy has become an invaluable tool to noninvasively interrogate the membrane architecture of bacteria to study the spatial organization of proteins associated with membranes, which in turn help us to understand how bacteria have evolved to exploit environmental niches. Model systems like *Escherichia coli* and *Caulobacter crescentus* have been used to study the spatiotemporal organization of membrane proteins. Like most gram-negative bacteria, the outer membrane of *E.coli* is populated with β -barrel proteins, which serve as selective channels where exchange of small molecules take place. Surface exposed domains in these channels provide means to fluorescently label and utilise them for fluorescent microscopy studies to investigate their spatial organization at the outer membrane. Here, we describe a methodology to fluorescently label outer membrane proteins in *E.coli* and study their spatial organization using direct stochastic optical reconstruction microscopy (*d*STORM).

Introduction

With the advent of super-resolution microscopy, a plethora of fluorescence microscopy techniques have come into existence enabling nanoscale scale imaging to interrogate, understand and redefine the subcellular localization of macromolecules in biological systems. To date, single molecule localization microscopy (SMLM) techniques including direct stochastic optical reconstruction microscopy (*d*STORM) have shown to reach lateral resolution of 10-25 nm (Kamiyama and Huang, 2012), a sufficient scale to directly visualize macromolecules in biological systems. Lately, these advanced fluorescent imaging techniques have been utilised to dissect membrane biology of prokaryotic systems (Xie et al., 2008). Model systems like *Escherichia coli* and *Caulobacter crescentus* have been used to study the spatiotemporal organization of membrane proteins in bacteria (Gahlmann and Moerner, 2014).

Gram-negative bacteria have a double membrane system (inner and outer membrane) containing integral and peripheral proteins alongside with phospholipids and glycolipids. Outer membrane in particular, displays an asymmetric architecture in which the inner leaflet is comprised of phospholipids and the outer leaflet decorated with lipopolysaccharides (LPS). Majority of outer membrane proteins have a β -barrel architecture (De Geyter et al., 2016; Plummer and Fleming, 2016; Noinaj et al., 2017; Slusky, 2017) and they often serve as selective channels for small molecules to move across in or out of the outer membrane. Surface exposed domains in these channels provide means to fluorescently label and utilise them for fluorescent microscopy studies to investigate their spatial organization, which in turn provide insights to understand the surface distribution of membrane proteins in bacteria (Gibbs et al., 2004; Spector et al., 2010; Rassam et al., 2015).

Here, we describe a methodology to fluorescently label outer membrane proteins in *E.coli* to study their spatial organization using single-molecule localization imaging technique, *d*STORM. This protocol can be readily extended to study the spatiotemporal organization of other membrane proteins beyond gram-negative bacterial systems.

Reagents

Reagents for bacterial culture

LB (1% tryptone, 0.5% yeast extract and 0.5% NaCl)

Antibiotics (100 mg/ml ampicillin, 30 mg/ml kanamycin and 50 mg/ml chloramphenicol)

Fluorophores for single colour *d*STORM imaging

Alexa Flour® 647 fluorescent dye (ThermoFisher®)

Sample preparation setup for *d*STORM

8-well plates cover glass bottom chambers (Sarstedt®)

0.01% (v/v) poly-L-lysine (Sigma-Aldrich®)

Phosphate buffered saline (PBS)

Paraformaldehyde (2% w/v)

Glutaraldehyde (0.2% v/v)

0.1% (w/v) NaBH₄ dissolved in PBS

Digitonin (0.001% or 0.01% w/v in PBS)

TritonX-100 (0.001% or 0.01% v/v in PBS)

5% w/v BSA (Sigma-Aldrich®)

Anti-mouse or Anti-rabbit IgG-Alexa-647 (ThermoFisher®)

TetraSpeck™ Microspheres, 0.1 μm (ThermoFisher® T7279)

dSTORM imaging buffer preparation

TN buffer (50 mM Tris-HCL, pH 8.0, 10 mM NaCl)

Oxygen scavenging system GLOX (0.5 mg/mL glucose oxidase; Sigma-Aldrich-G2133, 40 ug/mL catalase; Sigma-Aldrich-C-100 and 10% Glucose)

10 mM MEA (Sigma-Aldrich- M6500)

Equipment

Direct stochastic optical reconstruction microscopy (dSTORM) setup

Olympus IX-71 base equipped with the appropriate fluorescence filter cubes

488 nm Toptica laser (200 mW)

561 nm Quantum laser (500 mW)

638 nm Oxxius laser (150 mW)

UPlanSApo UIS2 oil-immersion 100x NA 1.4 objective

1.6x magnification changer engaged

Andor iXon Ultra 897 High Speed EMCCD camera

Procedure

Sample preparation for dSTORM

1. Grow *E.coli* cells to mid-log phase (O.D. 600 ~ 0.6) in LB media (Miller) at 37°C with shaking (200 rpm) in an Erlenmeyer flask.

Note - At Mid-log phase, protein synthesis and trafficking is at maximum to compensate the rapid cell growth.

2. Collect aliquots (500 μL) of cells by centrifugation (4000 x g, 5 min, 4°C) and then wash twice in PBS before being resuspended in 500 μL of PBS. Coat the cover glass bottom chambers of 8-well plates (Sarstedt®) with 0.01% (v/v) poly-L-lysine (Sigma-Aldrich®) for 10 min at room temperature and remove excess poly-L-lysine.

Note - It is necessary to optimise the concentration of poly-L-lysine, as higher concentrations would result in multilayers of bacteria, which hinders Total internal reflection fluorescence (TIRF) imaging.

3. Afterwards, immobilize 200 μ L of *E. coli* cells in to each coated well chamber. To ensure the formation of a monolayer of bacteria, centrifuge the chamber slides (4000 x g, 3 min, 4 ° C) and remove the excess cell suspension followed by three brief washes with PBS.

Note - Ensuring the formation of a monolayer of bacteria is critical to perform TIRF imaging.

4. Fix the monolayer of bacteria with a mixture of paraformaldehyde (2% w/v) and glutaraldehyde (0.2% v/v) in PBS for 5 min at 4°C followed by PBS washing steps to remove excess fixatives.

5. To minimise auto-fluorescence caused by Schiff's bases in glutaraldehyde, treat the samples with freshly prepared 0.1% (w/v) NaBH₄ dissolved in PBS for 15 min, followed by two washing steps with PBS.

6. Where applicable the samples can be permeabilized either with Digitonin (0.001% or 0.01% w/v in PBS) or TritonX-100 (0.001% or 0.01% v/v in PBS) followed by three washing steps with PBS.

Note - Depending on the localization of a particular protein in the cell, reagents for permeabilization may differ. For example, antibody accessibility to inner membrane environment can be increased by using TritonX-100, a much harsher detergent than digitonin.

Immunofluorescent labelling of *E.coli* membrane proteins

1. The specificity of an antibody should be tested with a negative control.

Note - In this instant, we have used a monoclonal antibody raised against *E.coli* membrane protein BamC. The absence of fluorescent signal on Δ bamC *E.coli* mutants confirms the specificity of anti-BamC antibody (Figure 1).

2. Blocking of non-specific binding sites is achieved by incubation in 5% w/v BSA in PBS for 1 h at room temperature, followed by incubation of antisera diluted to 1:1000 in 5% w/v BSA in PBS, for 1 h mixing by rotary inversion at room temperature.

3. Remove excess unbound primary antibodies by washing the samples thrice with PBS followed by addition of secondary anti-mouse IgG-Alexa 647 (ThermoFisher®) conjugated antibody (diluted 1:1000 in 5% BSA in PBS) for 45 min at room temperature. Follow the same washing steps to remove excess unbound secondary antibody. Store the samples at 4°C in PBS prior to microscopy imaging.

Note - Prior to imaging, a post fixation step can be carried out (with paraformaldehyde, 2% w/v) which ensures the preservation of antibodies in the sample for a long period of time.

4. Prior to imaging add fiducial markers (TetraSpeck™ Microspheres, 0.1 μ m ; ThermoFisher T7279) to the sample.

dSTORM image acquisition and processing

1. Collect super-resolution images for Alexa-647 labelled samples by illuminating continuously with 638 nm laser (1-5% laser power) at an appropriate TIRF angle.

Note - When performing dSTORM imaging, take necessary steps to adhere to laser safety guidelines enlisted in your imaging facility.

2. Drive dyes into the dark state (this takes about 30-60 s for Alexa-647, under 80-90% laser power), and then single molecule blinking time series can be captured. This can be under 10,000-20,000 frames at an exposure time of 20 ms and an electron multiplying gain of 50. Raw image pixel size with 100x objective and 1.6x magnifier engaged is 100 nm x 100 nm.

Note - Laser illumination parameters may change depending on the microscopy system and the type of fluorophore in use. Therefore it is paramount to optimise these parameters before acquiring images.

3. To reconstruct the series of to super-resolved images, an out-put pixel size of

10 nm is recommended. For this we have used the open-source software rapidSTORM version 3.3.1 (Wolter et al., 2012).

Note - You can also use other localization microscopy evaluation programs such as ThunderSTORM (<https://zitmen.github.io/thunderstorm/>) to achieve the desired resolution output.

4. Here, we have set the full width of half maximum (FWHM) of the fitting point spread function to 350 nm and fitted point spread functions (PSF) with a local signal-to-noise ratio (SNR) < 120. Images are first colour coded for temporal appearance of blinks to detect sample drift, then (if applicable) drift correction is achieved by using the linear drift correction available in rapidSTORM. If fiducial markers are used, use the fiducial based drift correction option in rapidSTORM. Finally, export the file as 8-bit greyscale images for further image processing.

5. Extract the corresponding localisations files for the single-molecule images from rapidSTORM software listing all detected localisations in x,y coordinates and use it for quantification and data analysis.

Cluster analysis of Super resolution images

Cluster analysis procedure can be found in the following link at Git repository; <https://github.com/monashmicroimaging/autoclustr>.

Troubleshooting

Time Taken

Anticipated Results

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Acknowledgements

This work was supported by the Multi-modal Australian Sciences Imaging and Visualisation Environment (MASSIVE; www.massive.org.au) and funded through an Australian Research Council (ARC) Laureate fellowship award (FL30100038), an ARC LIEF grant (LE150100110), and an NHMRC program grant (1092262). T.L. is an ARC Australian Laureate fellow, and S.D.G. is the recipient of an ARC Laureate postgraduate research scholarship.

Figures

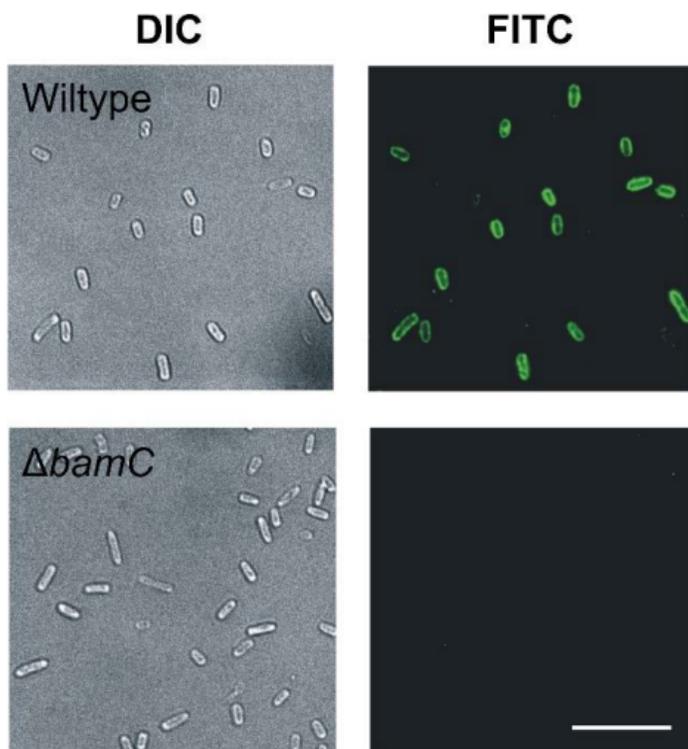


Figure 1

Immunofluorescence assessment of *E. coli* BW25113 using antibodies recognizing the C-terminal domain of BamC. At left, the differential interference contrast (DIC) image for a representative field of cells and at the right is the fluorescence image (FITC filter) of antibody staining. Also shown is a control experiment from an isogenic Δ bamC mutant strain that lacks BamC. Scale bar 10 μ m.

RapidSTORM reconstructed images

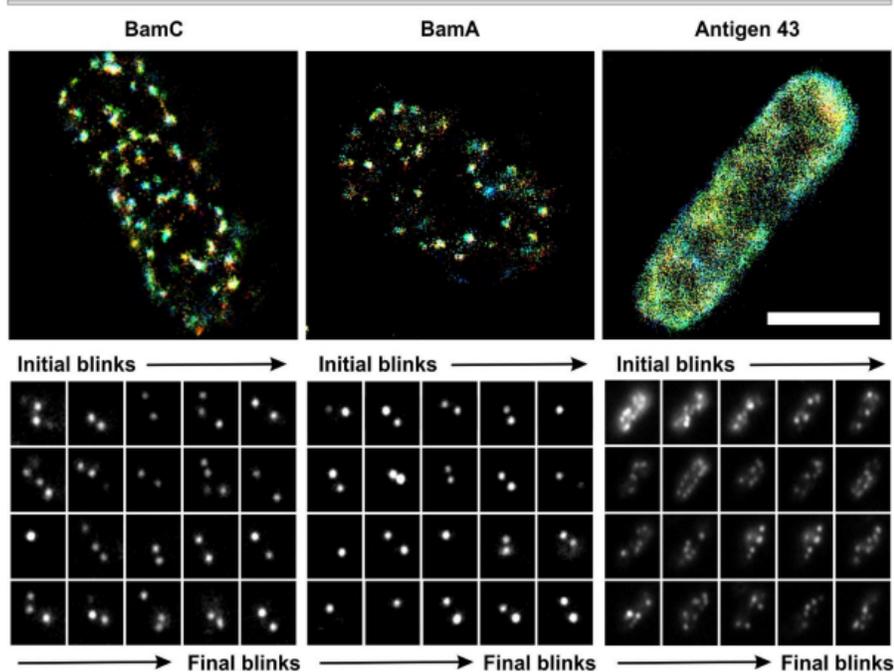


Figure 2

Photoswitching events captured through dSTORM. RapidSTORM reconstructed super-resolution images and the corresponding photoswitching events or "blink files" generated for BamC, BamA and Ag43 respectively (all outer membrane proteins in E.coli). Spatially well-separated localization events were acquired for 10,000 frames at an exposure of 20 ms. Scale bar 1 μm .