

Imaging the Cell Cycle of Pathogen *E. coli* During Growth in Macrophage

Gaëlle Demarre, Victoria Prudent, and Olivier Espéli

Abstract

The study of the bacterial cell cycle at the single cell level can not only give insights on the fitness of the bacterial population but also reveal heterogeneous behavior. Typically, the DNA replication, the cell division, and the nucleoid conformation are appropriate representatives of the bacterial cell cycle. Because bacteria rapidly adapt their growth rate to environmental changes, the measure of cell cycle parameters gives valuable insights for the study of bacterial stress response or host–pathogen interactions. Here we describe methods to first introduce fluorescent fusion proteins and fluorescent tag within the chromosome of pathogenic bacteria to study these cell cycle steps; then to follow them within macrophages using a confocal spinning disk microscope.

Key words *parS* sites, Microscopy, Fluorescence

1 Introduction

This method is dedicated to the investigation of the bacterial cell cycle during host infection, via the observation by fluorescence microscopy of chromosome tags, nucleoid-associated protein, and FtsZ ring formation. The bacterial cell cycle has been studied for decades in controlled conditions of laboratory growth. Most of the work has focused on growth in the absence of environmental stress. However, in their natural environment bacteria frequently encounter lack of nutrients and oxidative, genotoxic, or acidic stress. The progress of imaging methods allows for the evaluation of the bacterial cell cycle during host cell infection. The present protocol describes classical methods of cell cycle analysis by fluorescence microscopy adapted to the imaging of AIEC (Adherent Invasive *E. coli*) LF82 bacteria [1] during growth inside acidic phagolysosome of macrophages.

FROS (Fluorescent Repressor Operator Sequence) [2, 3] and *parS*/ParB-GFP [4] tags have been used to label specific loci and follow chromosome choreography [5, 6]; the number of foci

within a given cell is also a good indication of the availability of nutrients in the environments [7]. The labeling of the chromosome with the nucleoid associated protein HU fused with mCherry [8] is also an indicator of the environment encountered by the bacteria. The shape of bacterial nucleoid changes according to the nutrient used by the bacteria [9] and when genotoxic [10] or oxidative stress [11] is present. Finally cell division can be monitored by the observation of the main septal ring protein FtsZ [12, 13]. FtsZ polymerization is targeted by two cell cycle checkpoints, the SOS regulon and the Nucleoid Occlusion system that would modify the timing of division and provoke cell elongation [14, 15]. Therefore, imaging of the origin of replication, the nucleoid, and the septal ring are good indicators of the bacterial adaptation to an environmental challenge. We selected HU-mCherry, *oriC::parS*/ParB-GFP, and FtsZ-GFP fluorescent reporters because they produce robust signals that tolerate fixation and successive illumination during Z stack acquisition and can be accurately scored by image analysis tools such as Oufi [16], Fiji [17], or ObjectJ (<https://sils.fnwi.uva.nl/bcb/objectj/index.html>).

2 Material

2.1 Plasmids, Strains, and Primers

Human cell line:

THP1 (ATCC[®] TIB202[™]).

Bacterial Strains:

LF82 [18] Δbla (a generous gift from Nicolas Barnish).

MG1655 Hu-mcherry [19].

Plasmids:

pKOBEGA (pSC101, amp, TS) [20].

pCP20 (pSC101, amp, TS) [21].

pAD37 (R6k, cm) [22].

pFH2973 [23].

pFWZ5 [24].

Primers:

Construction of the HU-mCherry strain

hupA-up	AAGGATAACTTATGAACAAGACTC
hupA-down	AACAGTAATTGCGAACCTTCGG
hupA verif up	CGTCGCACTCGATGCTTAGC
hupA verif down	CCAGTGGATTTGCTGAAGACC

Insertion *parS_{pMT1}* (pAD37) (*FRT-aph-FRT-parS_{pMT1}*).

UpT1	5' < 50nt homology to <i>oriC</i> region (position-position) >CGG CTG ACA TGG GAA TTA GCC
DownT1	5' < 50nt homology to <i>oriC</i> region (position-position) >GGT CTG CTA TGT GGT GCT ATC T

2.2 Reagents: Stock Solutions

All solutions must be prepared using ultrapure water (by purifying deionized water, to attain a sensitivity of 18 MΩ-cm at 25 °C).

Prepare the following buffers and stock solutions. Unless otherwise specified, filter solutions using a 0.2 μm low protein binding nonpyrogenic membrane.

10% glycerol in H₂O cold.

Lennox broth (LB).

100 mg/ml ampicillin.

50 mg/ml kanamycin.

30 mg/ml chloramphenicol (pAD37).

20% arabinose.

1M IPTG.

2.3 Cell Culture

800 μg/ml PMA (phorbol 12-myristate 13-acetate).

RPMI 1640 without L-glutamine supplemented with 10% de-complemented fetal bovin serum and 2 mM glutamine (Life Technologies).

Keep at 4 °C no more than 1 month.

1× phosphate buffer saline (PBS).

24-well plate (1.9 cm², no coating).

2.4 Cell Fixation

Cold methanol kept at -20°C.

Fluorescence mounting medium: Dako S3023.

Microscope slides: 76 × 26 mm.

Microscope glass coverslips, 12 mm diameter, no coating.

2.5 Molecular Biology and Microbiology Material

Shaker: Infors Minitron.

Water bath.

Vacuum concentrator: miVac, Genevac.

PCR: C1000 Thermal cycler, Bio-Rad.

Electroporator: Micropulser, Bio-Rad.

2.6 Microscope

Inverted Zeiss Axio Imager with spinning disk CSU W1 (Yokogawa):
100× objective, NA = 1.4, oil, WD = 0.13 (WD: working distance in mm).

Camera ORCA-Flash 4.0 digital CMOS.

Lasers:

490 nm at 150 mW.

561 nm at 100 mW.

642 nm at 110 mW.

Metamorph Premier 7.6 software.

3 Methods
3.1 Strain Construction

AIEC LF82 will be genetically engineered to observe the nucleoid with HU-mCherry, the origin of replication with a *parS_{pMTI}* tag and the septal ring with a plasmid expressing FtsZ-GFP.

a. Transform AIEC LF82 with the plasmid pKOBEGA

Prepare competent bacteria using a protocol adapted for the strain of interest. For AIEC LF82, a culture of OD = 0.6 is washed 3 times in ice-cold 10% glycerol and the final resuspension volume is 1/250 of the initial volume.

Transform 50 µl of competent bacteria with 30 ng of pKOBEGA.

Plate at 30 °C on ampicillin (100 µg/ml).

b. Construction of AIEC LF82 HU-mCherry strain with λ Red (γ, β, *exo*) recombination [21]

b1. Prepare competent bacteria expressing λ Red (γ, β, *exo*) proteins as follows:

Dilute 500-fold an overnight culture of AIEC LF82 containing pKOBEGA in 50 ml LB (100 µg/ml amp, 0.15% arabinose).

Incubate with shaking at 30 °C up to OD_{600nm} = 0.6.

Incubate at 42 °C for 15 min.

Chill the culture in an ice-water bath for 15 min.

Centrifuge for 10 min at 5000× *g* at 4 °C.

Discard the supernatant and wash cell pellet with 25 ml of 10% cold glycerol.

Centrifuge for 10 min at 5000× *g* at 4 °C.

Wash cell pellet with 12 ml of 10% cold glycerol.

Centrifuge for 10 min at $5000\times g$ at $4\text{ }^{\circ}\text{C}$.

Wash cell pellet with 6 ml of 10% cold glycerol.

Centrifuge for 5 min at $5000\times g$ at $4\text{ }^{\circ}\text{C}$.

Resuspend the cell pellet in $150\text{ }\mu\text{l}$ 10% cold glycerol.

b2. Transform with *hupA-mcherry* PCR product prepared as follows

Perform 10 PCR of $50\text{ }\mu\text{l}$:

PCR Mix for $50\text{ }\mu\text{l}$.

$5\text{ }\mu\text{l}$ $10\times$ Extaq buffer.

$5\text{ }\mu\text{l}$ dNTP mix (2 mM each).

50 ng of genomic DNA (strain MG1655HU-mcherry).

$2.5\text{ }\mu\text{l}$ of $10\text{ }\mu\text{M}$ primer hupA-up.

$2.5\text{ }\mu\text{l}$ of $10\text{ }\mu\text{M}$ primer hupA-down.

1 unit Extaq Polymerase.

$33.7\text{ }\mu\text{l}$ H_2O .

PCR conditions:

Initial denaturation:

$98\text{ }^{\circ}\text{C}$ —3 min.

30 cycles:

$98\text{ }^{\circ}\text{C}$ —10 s.

$57\text{ }^{\circ}\text{C}$ —30 s.

$72\text{ }^{\circ}\text{C}$ —1 min/kb.

Final extension:

$72\text{ }^{\circ}\text{C}$ —10 min.

Hold:

$12\text{ }^{\circ}\text{C}$.

The expected fragment size is about 3 kb.

Concentration of PCR product:

Use PCR purification kit to clean the DNA (three columns for 10 PCRs, elution with $100\text{ }\mu\text{l}$ elution buffer per column); pool the three elutions in one tube.

Concentrate the DNA with a vacuum concentrator at $45\text{ }^{\circ}\text{C}$ up to a final volume of $30\text{ }\mu\text{l}$.

Dialyze 30 min on filter membrane (Millipore $0.02\text{ }\mu\text{m}$) floating on a petri dish filled with ultrapure water.

Electrotransformation of the PCR product:

Preincubate the electroporation cuvette (2 mm) in ice.

In a microcentrifuge tube mix ~5 µg of PCR product with 90 µl of the electrocompetent bacteria.

Transfer the mix to the precooled cuvette. Keep on ice.

Dry the cuvette. Insert it in the electroporator and immediately pulse at 2500 V.

Immediately add 1 ml of LB. Mix thoroughly with the bacteria and transfer to a 2 ml tube.

Incubate for 1 h at 37 °C with shaking.

Plate 100 µl of the transformation on 75 µg/ml kanamycin plate (*see Note 1*).

Centrifuge for 2 min the rest of the transformation tube.

Remove the supernatant.

Resuspend the cell pellet with 100 µl of LB, plate it on 75 µg/ml kanamycin plate.

Incubate overnight at 37 °C.

c. Delete the kanamycin gene through the flipase/FRT recombination

When it is required, the kanamycin resistance gene can be removed with Flp recombination using the protocol described in [21]. Choose kn^S and amp^S colonies.

d. Construction of AIEC LF82 HU-mCherry strain with a $parS_{pMTI}$ tag near *oriC*

Prepare $parS_{pMTI}$ product by PCR as described in paragraph b2 with 1ng of pAD37 as template and the primers UpT1 and downT1. The size of the expected fragment is about 1.7 kb.

Transform the AIEC LF82 HU-mcherry strain with the pKO-BEGA plasmid.

Prepare AIEC LF82 HU-mcherry pKOBEGA competent bacteria expressing lambda red (beta exo and gam) proteins as described in the paragraph b2.

Transform the strain HU-mcherry *oriC::parS_{pMTI}-FRT-aph-FRT* with the pFH2973. Select transformants on 100 µg/ml ampicillin plates.

e. Construction of AIEC LF82 HU-mCherry strain with a plasmid expressing FtsZ-GFP

Transform the AIEC LF82 HU-mcherry strain with the pFWZ5 plasmid.

3.2 Infection

a. Differentiation of THP1 monocytes in macrophages (*see Note 2*)

Prepare a 5×10^5 cell/ml cell suspension in the cell growth medium.

Just before use, dilute 5 μl of the 800 μM stock of PMA in 195 μl of growth medium (*see Note 3*).

Add diluted PMA at a 1000 \times dilution into the cell suspension.

Introduce a circular coverslip in each well.

Load 1 ml of cell suspension in each well. Remove the bubbles from under the coverslip by softly pressing on the coverslip with a pipette

Incubate for 18 h at 37 $^{\circ}\text{C}$ with 5% CO_2 .

b. AIEC LF82 culture

Growth the AIEC LF82 strain of interest overnight in LB supplemented with 100 $\mu\text{g}/\text{ml}$ ampicillin and 2 μM IPTG (to induce ParB-GFP from pFH2973). Measure $\text{OD}_{600\text{nm}}$, it should be close to 4.

Dilute them in 1 \times PBS up to an $\text{OD}_{600\text{nm}}$ of 0.4.

c. Infection

Check the THP1 cells; they should form heaps (*see Note 4*).

Wash the differentiated macrophages with 1 \times PBS. Eliminate the medium by inverting the plate over a trash bin and then above an absorbing paper.

Add 1 \times PBS drop by drop on the wall of the well.

Mix gently by rotation.

Add 1 ml of growth medium.

Add 78 μl of the bacterial suspension.

Mix gently by rotation.

Centrifuge for 10 min at 900 rpm at room temperature.

Incubate for 10 min at 37 $^{\circ}\text{C}$ with 5% CO_2 .

Wash twice with 1 \times PBS.

After the second wash, remove the last drop of PBS with a micropipette.

Add 1 ml of cell growth medium supplemented with 50 $\mu\text{g}/\text{ml}$ gentamycin in each well.

Incubate for 40 min or 24 h at 37 $^{\circ}\text{C}$ with 5% CO_2 .

3.3 Cell Fixation and Slide Preparation

a. Fixation of infected cells

At the selected time point the cell should be fixed before imaging.

Wash twice with 1 ml of 1 \times PBS.

Add 1 ml of 1 \times PBS and slowly add 1 ml of methanol (kept at -20°C) (1 Vol methanol/1 Vol PBS) (*see Note 5*).

Immediately remove the PBS + methanol mix.
 Add very slowly 1 ml of methanol.
 Incubate for 5 min on ice.
 Keeping the methanol add very slowly 1 ml of 1× PBS.
 Remove the PBS + methanol mix.
 Add very slowly 1 ml of 1× PBS.
 Keep in PBS at 4 °C till mounting.

b. Slide mounting

Load one drop (10 µl) of Dako on the microscope slide.
 Remove the coverslip from the plate with a pair of pliers.
 Damp the coverslip in fresh 1× PBS.
 Drain the PBS for the coverslip holding the coverslip with the pliers.
 Dry the coverslip by touching an absorbing paper with the edge of the coverslip.
 Transfer the coverslip onto the Dako drop, the side of the coverslip with the cell/bacteria should face the slide.
 Incubate overnight at room temperature in the dark.

3.4 Imaging and Image Processing

Imaging is performed on a Zeiss Axio imager microscope equipped with Yokogawa W1 spinning disk confocal head. Routinely, Z stacks of 36 images with a step of 0.4 are recorded to observe the complete macrophage volume. Laser excitations are performed at 488 nm for 600 ms at 80%, 561 nm for 200 ms at 20%, phase contrast for 50 ms. Image acquisition is controlled by Metamorph software.

Images are processed with Fiji for background subtraction, Z projection, Z plane color coding, and 3D reconstructions (Fig. 1). Object counting can be performed in 2D before or after Z projection with Fiji, Oufiti, or ObjectJ. Three-dimensional object counting and measurements can be performed after deconvolution (Huygens, Scientific Volume Imaging) with a 3D reconstruction and analysis tool (Imaris, Bitplane).

4 Notes

1. LF82 transformant selection on kanamycin plates required a kanamycin concentration of 75 µg/ml. This concentration may need to be adjusted in order to avoid false positive and to allow the kanamycin resistance from a single chromosomal copy of the kanamycin resistance gene.

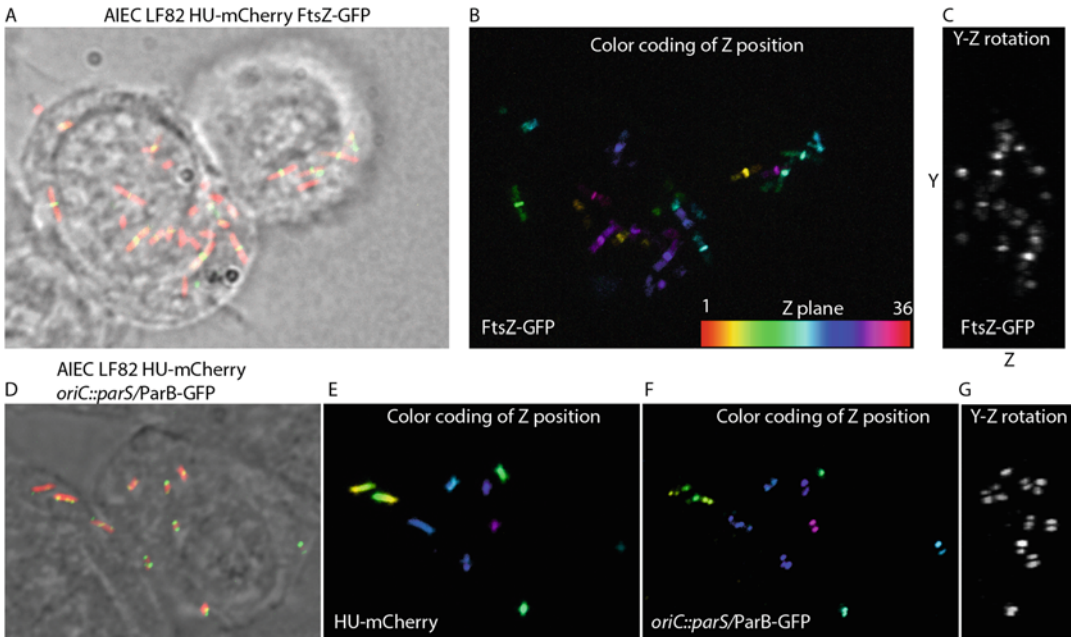


Fig. 1 (a) Representative image of AIEC LF82 HU-mCherry FtsZ-GFP, HU-mCherry (red), FtsZ-GFP (green). The image corresponds to the Z projection (sum of all planes) for the mCherry and GFP signals merged with the best focus plane of macrophage image. (b) Z plane color coding of the FtsZ-GFP signal. (c) 3D reconstruction and rotation along the YZ axis of the FtsZ-GFP signal. (d) Representative image of AIEC LF82 HU-mCherry *oriC::parS/ParB*-GFP, HU-mCherry (red), *oriC::parS/ParB*-GFP (green). The image corresponds to the Z projection (sum of all planes) for the mCherry and GFP signals merged with the best focus plane of macrophage image. (e) Z plane color coding of HU-mCherry signal. (f) Z plane color coding of *oriC::parS/ParB*-GFP signal. (g) 3D reconstruction and rotation along the YZ axis of the *oriC::parS/ParB*-GFP signal

2. One to three-week-old THP1 monocytes are differentiated within macrophages.
3. Do not introduce a micropipette in the growth medium bottle. Transfer with a pipette a small volume of the medium in an eppendorf tube. Pipette the 195 μ l from the eppendorf tube.
4. If the macrophages do not form heaps, they are not adherent and thus not differentiated.
5. Methanol and PBS are added very slowly to avoid detachment of the cells.

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