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Mark C. Leake *Editor*

Biophysics of Infection

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Biophysics of Infection

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Preface

Traditional insight into the process of infection has stemmed largely either from conventional bulk ensemble average techniques in vitro or from imaging methods either fixed tissue samples or on living cells but restricted to standard limits of optical resolution. However, recently many state-of-the-art interdisciplinary techniques of modern biophysics have emerged which enable us to understand details of the mechanisms of infection far more clearly than before. Essentially, many of these new methods enhance both the spatial and temporal resolutions of data acquisition. This has enabled us to probe *dynamic* processes of infection directly, and at a precision comparable to the molecular length scale of the key processes involved. These emerging interfacial tools of biophysics include, for example, a range of single-molecule biophysics methods as well as super-resolution microscopy techniques. This volume of The Biophysics of Infection, in the Advances in Experimental Medicine and Biology series includes new protocols, reviews and original research articles for such emerging experimental and theoretical approaches, which have resulted in a substantial improvement to our understanding of the complex processes of infection.

December 2015 Mark C. Leake

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Chapter 1
The Biophysics of Infection

Mark C. Leake

Abstract Our understanding of the processes involved in infection has grown enormously in the past decade due in part to emerging methods of biophysics. This new insight has been enabled through advances in interdisciplinary experimental technologies and theoretical methods at the cutting-edge interface of the life and physical sciences. For example, this has involved several state-of-the-art biophysical tools used in conjunction with molecular and cell biology approaches, which enable investigation of infection in living cells. There are also new, emerging interfacial science tools which enable significant improvements to the resolution of quantitative measurements both in space and time. These include single-molecule biophysics methods and super-resolution microscopy approaches. These new technological tools in particular have underpinned much new understanding of dynamic processes of infection at a molecular length scale. Also, there are many valuable advances made recently in theoretical approaches of biophysics which enable advances in predictive modelling to generate new understanding of infection. Here, I discuss these advances, and take stock on our knowledge of the biophysics of infection and discuss where future advances may lead.

Keywords Single-molecule biophysics · Super-resolution

This volume in the Advances in Experimental Medicine and Biology series consists of a collection of truly cutting-edge research studies, laboratory protocols, experimental and theoretical biophysical techniques and applications in use today by some of the leading international experts in the field of infection research. A key difference in emphasis with this volume compared with other earlier themed collections of infection research is on the emphasis on the utility of *interfacial methods,* which increase the underlying physiological relevance of infection investigation. These developments are manifested through applying methods such as single-molecule

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cellular biophysics which strive to maintain the native physiological context through investigation of living cells (Leake 2013), especially experimental methods using emerging tools of optical microscopy (Wollman et al. 2015), as well as methods which combine in vivo, in vitro and computational approaches to probe biological process such as the interaction of proteins with DNA (Wollman et al. 2015), such as the use of fluorescence microscopy methods to probe functional, living cells, especially so using microbial systems as model organisms (Lenn et al. 2008; Plank et al. 2009; Chiu and Leake 2011; Robson et al. 2013; Bryan et al. 2014; Llorente-Garcia et al. 2014; Reyes-Lamothe et al. 2010; Badrinarayanan et al. 2012; Wollman and Leake 2015; Lenn and Leake 2015; Cordes et al. 2015). The length scale of precision of experimental protocols in this area has improved dramatically over recent years and many cutting-edge methods now utilize state-of-the-art single-molecule approaches, to enable imaging of biomolecule structure to a precision better than the standard optical resolution limit (Miller et al. 2015), as well as emerging biophysics tools which use single-molecule force spectroscopy (Leake et al. 2003, 2004, 2006; Linke and Leake 2004; Bullard et al. 2006). This volume also includes more complex representative methods to investigate infection through the use of advanced mathematical analysis and computation.

It is clear is that combining pioneering molecular biology, biochemistry, structural biology and genetics methods with emerging, exciting tools from the *younger* areas of biophysics, bioengineering, computer science and biomathematics, that our understanding of the processes of infection are being transformed. Improvements in all of these fields are likely to add yet more insight over the next years in the near future into the complex interactions between multiple key molecular players involved in infection.

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Chapter 2
Single-Molecule Observation of DNA Replication Repair Pathways in *E. coli*

Adam J.M. Wollman, Aisha H. Syeda, Peter McGlynn and Mark C. Leake

Abstract The method of action of many antibiotics is to interfere with DNA replication—quinolones trap DNA gyrase and topoisomerase proteins onto DNA while metronidazole causes single- and double-stranded breaks in DNA. To understand how bacteria respond to these drugs, it is important to understand the repair processes utilised when DNA replication is blocked. We have used tandem *lac* operators inserted into the chromosome bound by fluorescently labelled *lac* repressors as a model protein block to replication in *E. coli*. We have used dual-colour, alternating-laser, single-molecule narrowfield microscopy to quantify the amount of operator at the block and simultaneously image fluorescently labelled DNA polymerase. We anticipate use of this system as a quantitative platform to study replication stalling and repair proteins.

Keywords Single-molecule · Super-resolution · Fluorescent protein · In vivo imaging · DNA repair

2.1 Introduction

2.1.1 Antibiotics Interfere with DNA Replication

Different types of antibiotics kill bacteria by interfering with DNA replication. In bacteria, a sophisticated complex of protein machinery, called the replisome, replicates DNA by unwrapping its double helix and using the two exposed single strands as templates for DNA synthesis creating a structure called the replication fork (Reyes-Lamothe et al. 2010). Failure to copy DNA completely or accurately results in potentially disastrous consequences for the cell. The antibiotic family of Quinolones bind to two bacterial complexes associated with DNA replication, DNA

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gyrase and topoisomerase IV (Mustaev et al. 2014). These complexes remove positive DNA supercoils generated by the replisome and also disentangle intertwined sister chromosomes as replication proceeds. These reactions occur by binding of gyrase or topoisomerase IV to one DNA duplex, cleavage of that duplex and passage of another region of the chromosome through the break prior to sealing the break to reform an intact chromosome. The outcome of this complex reaction is the release of torsional strain or chromosome disentanglement (Drlica and Zhao 1997). Quinolones trap these topoisomerases on the DNA by stabilising a covalent protein-DNA complex that is a normal part of the reaction cycle for both gyrase and topoisomerase IV, generating a protein block to replication and disrupting the DNA architecture (Mustaev et al. 2014). Another antibiotic, metronidazole, disrupts replication by inducing single-strand and double-strand breaks on DNA in anaerobic pathogens (Edwards 1977). Metronidazole is readily reduced, creating DNA damaging compounds in anaerobes but is easily re-oxidised in aerobes. There is evidence of increased DNA repair in *Helicobacter pylori* when exposed to metronidazole (Goodwin et al. 1998).

2.1.2 Replisome Response to Blocks

DNA damage occurs naturally in *E. coli*, due to reactive oxygen species, chemicals and radiation causing double- and single- stranded breaks on DNA. There are also natural protein blocks to replication. Transcription occurs concurrently with DNA replication and as RNA polymerases are an order of magnitude slower than replisomes in bacteria, collisions can occur (McGlynn et al. 2012). RNA polymerases can also become stalled on the DNA by template damage, leading to the build-up of many polymerases (McGlynn et al. 2012). Thus, the replisome encounters many blocks to replication during the normal cell cycle and has been shown to pause frequently (Gupta et al. 2013). Many of these stalled replisomes can continue if the block is removed, which is advantageous, as reloading the replisome can lead to genome rearrangements (Syeda et al. 2014). However, replisomes lose functionality over time (Yeeles and Marians 2011) and so replisome reloading mechanisms are required, for when replisome barriers are not cleared sufficiently rapidly, prior to the blocked replisome losing activity (Duch et al. 2013).

DNA replication is initiated from *oriC* in a sequence-specific manner on the genome. However, the replisome can stall anywhere and so different reloading and re-initiation mechanisms are required for stalled replication forks that are DNA structure-rather than DNA sequence-specific. These mechanisms are not fully understood. Two proteins, PriA and PriC, can both reload the replicative helicase DnaB back onto replication fork structures. DnaB plays a central role in the replisome, unwinding the two DNA template strands and also acting as an organising hub for the entire replisome complex. PriA and PriC recognise different forked DNA structures that together represent all possible types of fork structure on the chromosome (Yeeles et al. 2013). *priA* and *priC* can be separately deleted from

the genome but a knock-out mutant of both is not viable, thus these repair pathways are essential for cell survival. The in vivo dynamics of these proteins are unknown and there is evidence that DNA at forks needs processing by other proteins to allow repair or bypassing the block (Lecointe et al. 2007; Atkinson and McGlynn 2009). There is also an accessory helicase, Rep, which promotes the movement of replisomes through protein blockages on DNA (Guy et al. 2009; Boubakri et al. 2010).

It is therefore important to study blocks to replication not only to understand the effect of antibiotics but also to understand how DNA replication is successfully completed in the face of the many natural blocks to replisomes inside cells. To study stalled replication, we have used a model protein block to replication by inserting tandem binding sites (34 copies of *lacO*) for the *lacI* transcription factor into the *E. coli* genome and over expressing the LacI protein. The Lac repressor-operator complex mimics naturally occurring protein-DNA complexes and inhibits fork movement with an affinity typically encountered during genome duplication. Since the majority of forks continue through a single complex unhindered, multiple complexes are required to give detectable inhibition of fork movement (Payne et al. 2006; Guy et al. 2009). Studying replisomes stalled at these blocks is an ideal problem for single-molecule microscopy, as it requires observation of individual replication machineries at blocks in the natural cell environment and also the associated repair proteins.

2.1.3 Single-Molecule Fluorescence Microscopy

Fluorescent protein fusions can act as reporters to provide significant insight into a wide range of biological processes and molecular machines. They can be used to gain insight into stoichiometry and architecture as well as details of molecular mobility inside living, functional cells with their native physiological context intact (Lenn et al. 2008; Plank et al. 2009; Chiu and Leake 2011; Robson et al. 2013; Llorente-Garcia et al. 2014; Bryan et al. 2014; Corbes et al. 2015). These fusion proteins can be used in conjunction with single-molecule narrowfield microscopy, and its similar counterpart Slimfield microscopy, as a versatile tool to investigate a diverse range of protein dynamics in live cells to generate enormous insight into biological processes at the single-molecule level. It has been used in *E. coli* to investigate DNA replication by determining the stoichiometry of the components of the bacterial replisome (Reyes-Lamothe et al. 2010) and the proteins involved in the structural maintenance of chromosomes (Badrinarayanan et al. 2012).

In narrowfield microscopy, the normal fluorescence excitation field is reduced to encompass only a single cell and produce a Gaussian excitation field (~20 μm^2) with 100–1000 times the laser excitation intensity of standard epifluorescence microscopy. Using such intense illumination causes fluorophores to emit many more photons, greatly increasing the signal to noise. This allows millisecond timescale imaging of individual fluorescently labelled proteins in their native

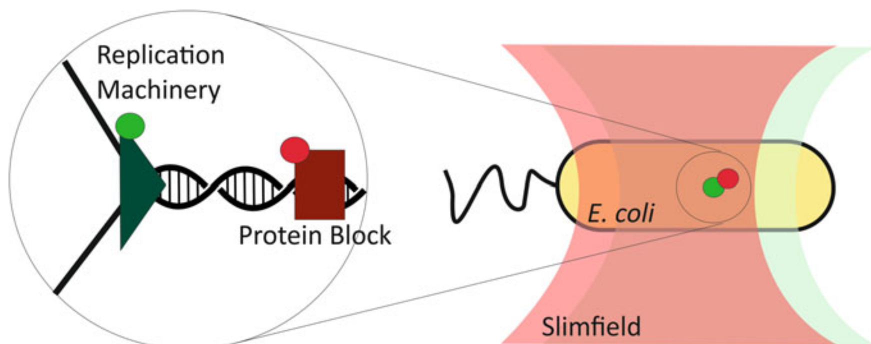

Fig. 2.1 Schematic of slimfield observation of fluorescently labelled replisome components encountering a fluorescently labelled protein block in *E. coli*

cellular environment. This time scale is fast enough to observe the diffusional motion of proteins and the dynamics which may occur around the replication fork.

We have labelled the *lac* operator replication block with a fluorescent *lac* repressor-mCherry fusion protein together with the dnaQ replisome component fused to the monomeric green fluorescent protein (GFP) allowing simultaneous imaging of the replisome and block. Using a bespoke narrowfield microscope, we have observed complexes of these proteins in live cells (see schematic in Fig. 2.1). To reduce the impact of autofluorescence caused by the blue GFP-excitation light, we have used high speed alternating-laser excitation (ALEX) to alternately excite each fluorophore at high speed. This enables the relatively dim mCherry protein to be observed without autofluorescence contamination and co-localised with GFP at high speed. Using custom software (Miller et al. 2015; Wollman et al. 2015a), we can quantify the number of fluorescently labelled proteins present in molecular complexes. Here, we demonstrate quantification of a replisome component and model protein replication block and show simultaneous imaging of both in the same live cell.

2.2 Methods

2.2.1 Generating Fluorescent Strains

2.2.1.1 Construction of Chromosomal *dnaQ-mGFP* Fusion

To create a *dnaQ-mGFP* C-terminal fusion, a PCR fragment containing mGFP and a downstream kanamycin resistance cassette amplified from pDHL580 (Landgraf et al. 2012) using primers oAS77 and oAS78 was recombineered as described (Datsenko and Wanner 2000) immediately downstream of *dnaQ* into PM300, a derivative of MG1655. The recombinants were selected for kanamycin resistance

and successful integration was confirmed by PCR and subsequent sequencing. The verified strain was called AS217.

2.2.1.2 Construction of *LacI-mCherry* Fusion

A synthetic *lacI-mCherry* C-terminal fusion from plasmid pAS13 (Eurofins MWG operon synthesis) was subcloned into pBAD24 between *Nco*I and *Xba*I sites that placed it under the control of the arabinose inducible promoter. The presence and orientation of the construct was confirmed by restriction digestion, sequencing and phenotypic testing in a reporter strain. The verified plasmid was called pAS17.

2.2.1.3 Construction of Strain with Lac Repressor Array, *dnaQ-mGFP* and *LacI-mCherry*

dnaQ-mGFP was moved from AS217 to a laboratory stock strain AS249 carrying *lacO*$_{34}$ (Payne et al. 2006) by phage P1-mediated transduction. The transductants were selected for kanamycin resistance and presence of the *dnaQ-mGFP* allele was confirmed by PCR. The resulting *lacO*$_{34}$ *dnaQ-mGFP* strain (AS271) was transformed with pAS17 to create a dual-labelled strain with an inducible roadblock to replication.

2.2.2 Growing Strains and Inducing the Lac Repressor

Single colonies from transformation of AS271 with pAS17 were grown in 5 ml Luria-Bertani (LB) ampicillin and Isopropyl-β-D-thiogalactopyranoside (IPTG) in 15 ml culture tubes overnight. 1 ml of the overnight culture was washed twice with 1X 56 salts and inoculated into 10 ml 1X 56 salts together with ampicillin, glucose for growth and arabinose for Lac repressor induction and grown to an A_{650} of 0.4–0.6 (mid log phase). Concentrations of ampicillin, glucose, arabinose and IPTG were 100 µg/ml, 0.1 %, 0.02 % and 1 mM respectively. Cells from 1 ml of culture were resuspended in 100 µl of fresh 1X 56 salts medium for visualisation.

2.2.3 Fluorescence Microscopy

2.2.3.1 The Microscope

Our bespoke inverted fluorescence microscope was constructed from a Zeiss microscope body using a 100x TIRF 1.49 NA Olympus oil immersion objective lens and a *xyz* nano positioning stage (Nanodrive, Mad City Labs). Fluorescence

excitation used 50 mW Obis 488 and 561 nm lasers, modulated using TTL pulses sent from National Instruments digital modulation USB module. A dual pass GFP/mCherry dichroic with 25 nm transmission windows centred on 525 and 625 nm was used underneath the objective lens turret. The beam was reduced 0.5x, to generate an excitation field of intensity ~6 Wcm^{-2}. The beam intensity profile was measured directly by raster scanning in the focal plane while imaging a sample of fluorescent beads. A high speed camera (iXon DV860-BI, Andor Technology, UK) was used to image at 5 ms/frame with the magnification set at ~50 nm per pixel. Laser emission was modulated such that each laser was on for 5 ms in alternating frames to give a 10 ms sampling time with 5 ms exposure time. The camera CCD was split between a GFP and mCherry channel using a bespoke colour splitter consisting of a dichroic centred at pass wavelength 560 nm and emission filters with 25 nm bandwidths centred at 525 and 594 nm. The microscope was controlled using our in-house bespoke LabVIEW (National Instruments) software.

2.2.3.2 Preparing Samples and Obtaining Fluorescence Data

E. coli cells were imaged on agarose pads suffused with media (Reyes-Lamothe et al. 2010). In brief, gene frames (Life Technologies) were stuck to a glass microscope slide to form a well and 500 µl 56 salts media plus 1 % agarose was pipetted into the well. The pad was left to dry at room temperature before 5 µl *E. coli* culture was pipetted in 6–10 droplets onto the pad. This was covered with a plasma-cleaned glass coverslip and imaged immediately. For each sample 10–30 cells were imaged in fluorescence and brightfield.

2.2.4 Analysing the Data

Single fluorescent proteins or complexes of proteins can be considered point sources of light and so appear as spatially extended spots in a fluorescence image due to diffraction by the microscope optics (Wollman et al. 2015b). Narrowfield fluorescence microscopy data consists of a time-series of images of spots which require *in silico* analysis to track each spot. We used custom Matlab™ tracking software to automatically identify spots, quantify them and link them into trajectories (Miller et al. 2015; Wollman et al. 2015a). The software identifies candidate bright spots using a combination of pixel intensity thresholding and image transformation. The threshold is set using the pixel intensity histogram as the full width half maximum of the peak in the histogram which corresponds to background pixels. A series of morphological transformations including erosion and dilation is applied to the thresholded image to remove individual bright pixels due to noise and

leave a single pixel at each candidate spot co-ordinate. The intensity centroid of candidate spots is found using iterative Gaussian masking (Thompson et al. 2002) and the characteristic intensity is defined as the sum of the pixel intensities inside a 5 pixel radius region of interest around the spot minus the local background (Xue and Leake 2009) and corrected for non-uniformity in the excitation field. If this spot is above a pre-set signal to noise ratio, defined as the characteristic intensity divided by the standard deviation of the local background, it is accepted. Trajectories are formed by linking together spots in adjacent frames based on their proximity and intensity.

The number of fluorophores present in a molecular complex is determined by dividing its intensity by the intensity of a single fluorophore. The characteristic intensity of a single fluorophore can either be determined from in vitro measurements of purified fluorophore or from the in vivo data itself using the intensity of spots found after bleaching the cell.

2.3 Results and Discussion

2.3.1 Quantifying dnaQ

The single labelled dnaQ-GFP strain was imaged using narrowfield microscopy. An example cell is shown in Fig. 2.2. The brightfield image of the cell is shown in Fig. 2.2a and the fluorescence image of dnaQ-GFP shown in Fig. 2.2b. Two spots of dnaQ can be seen in the fluorescence image corresponding to the two copies of the replisome, consistent with previous observations (Reyes-Lamothe et al. 2010). Spots found by software over all frames are shown as green and blue circles in Fig. 2.2a with their intensity values plotted against time in Fig. 2.2c in units of characteristic GFP intensity. The spots have a stoichiometry of 3 dnaQ-GFP per replisome, consistent with previous observations (Reyes-Lamothe et al. 2010).

The distribution of dnaQ replisome stoichiometries was obtained from a kernel density estimation and is shown in Fig. 2.3. The stoichiometry peaks at 2 and ranges up to 6 dnaQ per replisome. This agrees well with previous observations of 2–3 per replication fork. These forks appear to be overlapping when replication is initiated from the origin leading to the observation of double stoichiometries. These results combined with recent measures of the total copy number of dnaQ (Wollman and Leake 2015) are in good agreement with a previous study which labelled dnaQ with the Ypet fluorophore. It has been suggested that the fluorophore used in a fusion protein can effect the stoichiometry of native complexes (Landgraf et al. 2012), but here we observe no difference between Ypet and monomeric GFP fusions.

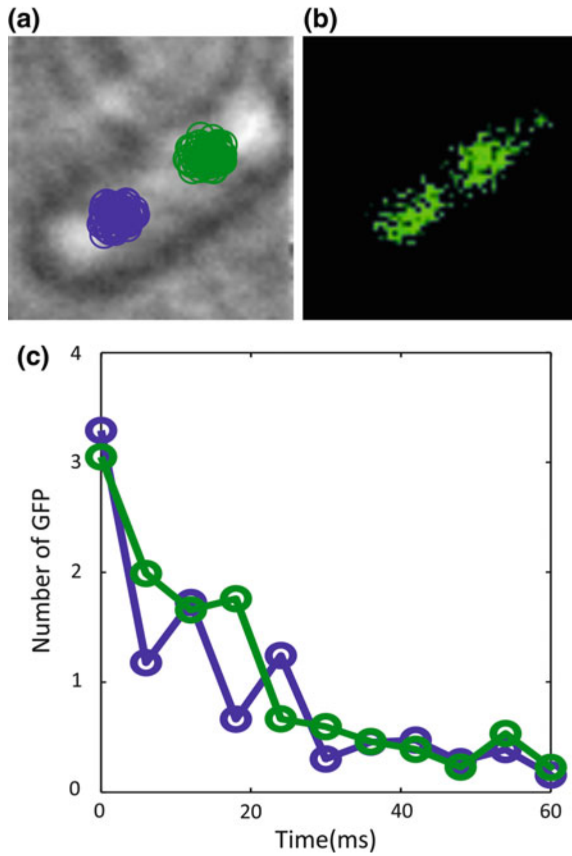

Fig. 2.2 **a** Brightfield image of an *E. coli* cell with tracked dnaQ-GFP overlaid, **b** fluorescence micrograph of dnaQ-GFP, **c** intensity of each spot over time in GFPs

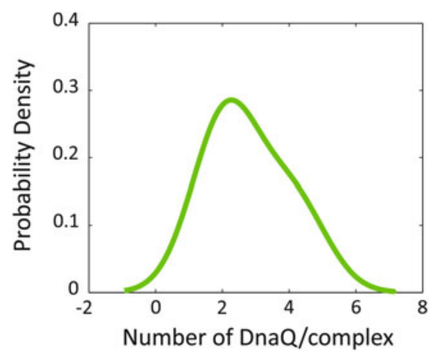

Fig. 2.3 Kernal density estimation of the number of dnaQ-GFP per spot

2.3.2 Quantifying LacI

We then imaged the *lac* operator blocks. This required optimisation of the growth conditions and expression levels as the fluorescently labelled *lac* protein is not endogenously expressed in this strain. Cells were grown in minimal media so that growth is slowed and there is, on average, only one replisome per cell. This not only eliminates the noise caused by LB autofluorescence but also the signal from multiple replisomes. Thus, having a single replisome greatly simplifies its tracking on the chromosome when it encounters the block and also makes downstream analysis easier by eliminating complexities due to multiple factors coming into play.

The results are shown in Fig. 2.4, with a brightfield image in Fig. 2.4a and mCherry fluorescence image in Fig. 2.4b. Two mCherry spots are seen in the fluorescence image, consistent with the *lac* operator sites having been replicated. All spots found over time are marked as red circles in Fig. 2.4a and their intensity plotted over time in Fig. 2.4c in units of mCherry intensities. The stoichiometry of the complexes is much lower than the 34 possible sites on the DNA and is closer to 5–10. This is unlikely to be caused by low expression levels as there is a significant diffuse background in the cell from unbound LacI-mCherry molecules. These results imply that the *lac* operators are not saturated with repressor. Further study is needed to understand the basis of this lack of saturation. The expression level could be varied and the number of potential binding sites on the DNA changed.

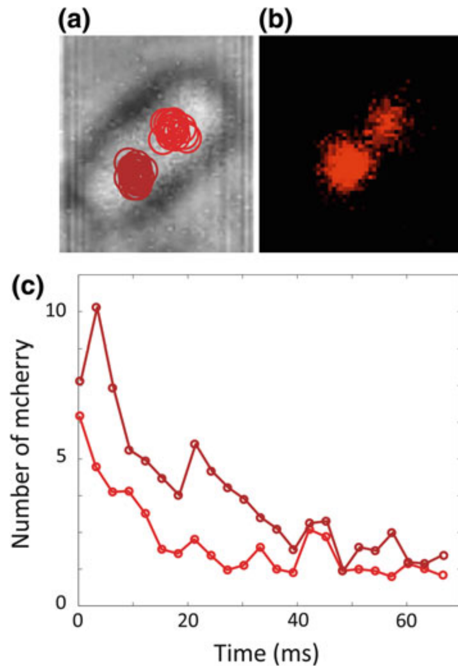

Fig. 2.4 **a** Brightfield image of an *E. coli* cell with tracked LacI-mCherry overlaid, **b** fluorescence micrograph of LacI-mcherry, **c** intensity of each spot over time in mcherrys

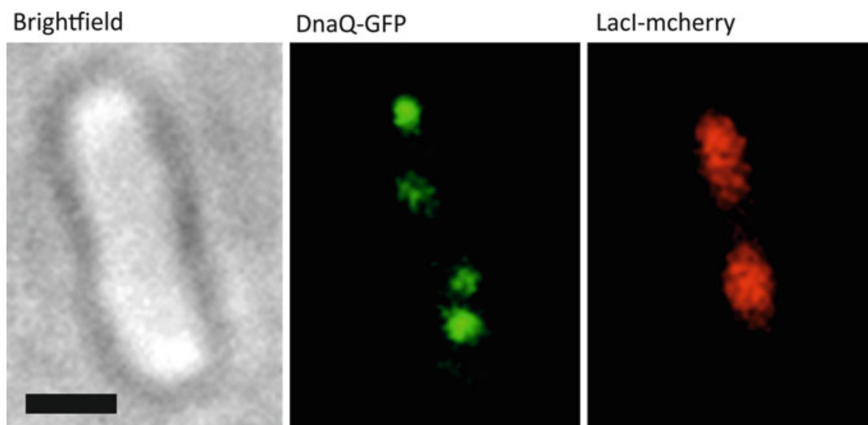

Fig. 2.5 *Left* brightfield image of an *E. coli* cell, *middle* fluorescence micrograph of dnaQ-GFP, *right* fluorescence micrograph of LacI-mCherry in the same cell

2.3.3 Dual-Colour Experiments

The *lac* operator block plus LacI-mCherry has been incorporated into the dnaQ-GFP strain and preliminary data obtained. Figure 2.5 shows brightfield and fluorescence micrographs of the dual-labelled strain. Our intention is to use this strain as a platform to study stalled replication by observing the behaviour of the replisome as it encounters different blocks with varying numbers of *lac* operators. This system could also be used to study repair proteins and could be combined with three colour microscopy, labelling the dnaQ with CyPet, the repair protein with Ypet and retaining the mCherry labelled *lac* operator array.

2.4 Summary

We have used tandem *lac* operators inserted into the chromosome bound by fluorescently labelled *lac* repressors as a model protein block to replication in *E. coli*. This block is a model for the action of some antibiotics such as quinolones which trap gyrases and topoisomerases on DNA. We have used dual-colour, alternating-laser, single-molecule narrowfield microscopy to quantify the amount of operator at the block and simultaneously image fluorescently labelled DNA polymerase. This quantitative platform for studying replication stalling will underpin future investigations.

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Chapter 3
Investigating the Swimming of Microbial Pathogens Using Digital Holography

K.L. Thornton, R.C. Findlay, P.B. Walrad and L.G. Wilson

Abstract To understand much of the behaviour of microbial pathogens, it is necessary to image living cells, their interactions with each other and with host cells. Species such as *Escherichia coli* are difficult subjects to image: they are typically microscopic, colourless and transparent. Traditional cell visualisation techniques such as fluorescent tagging or phase-contrast microscopy give excellent information on cell behaviour in two dimensions, but no information about cells moving in three dimensions. We review the use of digital holographic microscopy for three-dimensional imaging at high speeds, and demonstrate its use for capturing the shape and swimming behaviour of three important model pathogens: *E. coli*, *Plasmodium* spp. and *Leishmania* spp.

Keywords Optical microscopy · Holography · Image analysis · Leishmania · Plasmodium

3.1 Introduction

Microbial pathogens are responsible for the majority of annual mortality and morbidity. A high-profile example is malaria, which infects around 200 million people, primarily in the developing world (World Health Organization 2014).

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