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Visualizing genetic loci and molecular machines in living bacteria

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Abstract

An ongoing mission for biologists is to probe the molecular nature of cellular processes within live cells. Although much of what we have discovered during the molecular biology revolution of the last 50 years has been achieved by exploiting bacteria as 'bags of DNA and proteins', relatively little has been learnt about how they organize their life processes within cells. The mistaken perception of bacteria cells as unstructured systems arose partly because of the difficulty of performing studies by light microscopy due to their small size (many of them having cell lengths a few times bigger than the wavelength of visible light). With the opportunities provided by a range of new fluorophores and by new microscopic techniques, a revolution in bacterial cell biology is revealing unimagined organization in the bacterial cell. We review the development and exploitation of new visualization methods and reagents and show how they are contributing to the understanding of bacterial structure, chromosome organization, DNA metabolism and their relationship to the cell cycle.

Introduction

Although the existence of creatures too small to be seen by eye had long been suspected, their discovery was linked to the invention of the microscope. Bacteria were first observed by the Dutch amateur microscope builder Antony van Leeuwenhoek (1632–1723) in 1683 using a single-lens microscope of his own construction [1]. The process in understanding the nature of these tiny organisms came only very slowly due to their small size and the lack of techniques and reagents for probing their cellular organization.

For over 120 years, it has been possible to visualize the behaviour of eukaryotic chromosomes during segregation. As early as the 1880s, Walther Flemming (1842–1905) described in great clarity the chromosome behaviour during mitosis in animal cells [2]. Later, Theodor Boveri (1862–1915)'s work in sea urchins led to the emergence of the chromosome theory of heredity [3]. However, bacteria were generally regarded as pre-cellular in complexity and devoid of nuclei and other genetic apparatus of 'real' organisms in the first half of the twentieth century. In the 1950s and 1960s, electron microscopy was applied to bacterial cells and it was confirmed that bacteria were simple, lacking any membrane-bound organelles, especially a nucleus [4]. At around the same time, live-cell microscopy was applied to bacteria and the nucleoids could be visualized using gelatin-mounted slides without any staining [5,6].

In the 1970s, the birth of recombinant DNA technology and DNA sequencing led to a revolution in the understanding of genetics and their manipulability. In the 1990s, the successful application of immunofluorescent methods to visualize bacterial proteins, followed by the use of GFP (green fluorescent protein) as a tracer molecule for proteins [7] together with the development of digital imaging, has led to a new level of our understanding of the bacterial cell. The development of new fluorescent proteins with different emission spectra has made it possible to penetrate deeper into the mysteries of the cell by tracking multiple components in living cells at the same time [8]. Diverse techniques derived from fluorescence microscopy, such as FCS (fluorescence correlation spectroscopy) [9], FRAP (fluorescence recovery after photobleaching), FLIP (fluorescence loss in photobleaching), FRET (fluorescence resonance energy transfer) [10], TIRF (total internal reflection fluorescence) [11] and non-fluorescence techniques such as cryo-electron tomography [12], allow the determination of the numbers of molecules, diffusion rates, interaction with other proteins and their organization in complexes. This provides a novel approach to study cellular organization and processes in individual bacteria. In the present review, we focus on the development of epifluorescence techniques for the study of bacteria.

Methods for visualization of bacterial proteins and DNA in fixed cells

Immunofluorescence was developed to visualize proteins in eukaryotic cells in the 1940s [13] and was adapted to bacterial cells in the 1990s [14]. These methods use a fluorescent-labelled antibody to identify the localization of a target protein (Figure 1). In some cases, the primary antibody is directly labelled with fluorophores (Figure 1A). More commonly, an indirect method is performed using two sets of antibodies (Figure 1B). The primary antibody is used to recognize the

Key words: bacterium, chromosome, epifluorescence, genetic locus, green fluorescent protein (GFP), microscopy.

Abbreviations used: BrdU, bromodeoxyuridine; FISH, fluorescence *in situ* hybridization; FROS, fluorescent-repressor-operator system; GFP, green fluorescent protein.

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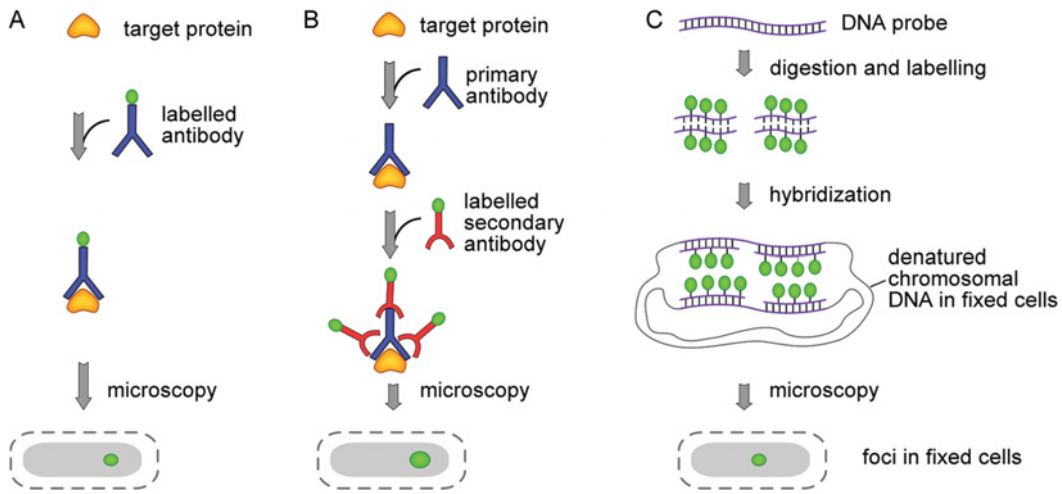
Figure 1 | Visualizing proteins and DNA in fixed cells

(**A**, **B**) Immunofluorescence. Cells are fixed, followed by immobilization and permeabilization on a slide. For direct immunofluorescence (**A**), cells are treated with fluorophore-labelled primary antibodies and visualized by fluorescence microscopy. For indirect immunofluorescence (**B**), cells are treated with primary antibodies, and a labelled secondary antibody to recognize the primary antibody. Thus the signal is amplified compared with direct immunofluorescence. (**C**) FISH. The amplified DNA fragments from PCR or cloning are digested into small fragments (50–200 nt) and labelled with fluorophores. These DNA probes are hybridized to denatured chromosomal DNA in fixed and permeabilized cells, followed by fluorescence microscopy.



protein of interest and a fluorophore-conjugated secondary antibody then recognizes the primary antibody. Usually, these antibodies are generated from different species. For example, the primary antibodies of several proteins of interest can be generated from one species, such as rabbit, and the same fluorescent-labelled goat-anti-rabbit antibody (secondary antibody) can be used to recognize the constant region of the rabbit antibody. In this case, the fluorescent-labelled secondary antibody can be used in multiple experiments and the fluorescent signal is usually enhanced, because a primary antibody can be bound by several secondary antibodies (Figure 1B).

Immunofluorescence can also be used to label newly replicated DNA [15]. Cells are first pulse-labelled with the nucleotide analogue BrdU (bromodeoxyuridine), which is incorporated into nascent DNA, and then gently fixed and permeabilized. An anti-BrdU antibody and a fluorophore-conjugated secondary antibody are used to detect the localization of newly replicated DNA.

FISH (fluorescence *in situ* hybridization) was developed for eukaryotes in the 1980s [16] and was later adapted to prokaryotes in the late 1990s [17]. It has now been very well developed and widely used to visualize specific chromosomal regions (Figure 1C). The DNA region of interest, usually 2–10 kb, is either amplified using PCR or cloned into a plasmid vector followed by restriction digestion. This DNA is further processed into FISH probes by digesting it into smaller pieces (<200 nt), denaturing and labelling it with fluorophores. These probes are then hybridized to the denatured chromosomal DNA inside fixed and permeabilized cells followed by detection by fluorescence microscopy.

A major advantage of these methods is that they can be done with wild-type cells without any genetic modification. For FISH, multiple loci can be detected at the same time using different fluorophores with no overlapping spectra [18]. The main disadvantage of these techniques is that they require the cells to be fixed and permeabilized to get labelled DNA and large antibody molecules into the cells without destroying them, which is technically demanding and can lead to an underestimate of the number of foci and to artefacts in visualization. Furthermore, since cells are fixed, these methods do not allow the study of dynamics of proteins and genetic loci.

Methods for visualization of proteins in live bacterial cells

GFP tagging is the clearest and most unequivocal way of showing the distribution and dynamics of proteins in living cells. Extensive site-directed mutagenesis has generated new fluorescent proteins with different emission spectra, which enable the simultaneous tracking of multiple components [8,19]. In bacteria, fluorescent fusions to the components of the replisome, divisome and cytoskeletal proteins are widely used to study the structure of the cell and important processes in cell function [20–22]. It is now clear that bacterial cytoskeletal elements, such as tubulin-like FtsZ and actin-like MreB, form protofilaments and play key roles in cell division, chromosome and plasmid segregation and the

maintenance of cell shape and polarity [23]. The bacterial cell is very well organized, with processes of DNA replication, segregation and cell division precisely choreographed [24].

Although the contribution of GFP to modern biology cannot be overemphasized, there are several limitations to its use. First of all, the cells need to be genetically modified to produce the protein with a ~30 kDa GFP fusion, which can compromise the full function of the target protein. Also, the dosage of the protein can be affected if the fusion protein is expressed as an ectopic copy in the chromosome or from a plasmid. This can lead to a misleading change in the pattern of localization from the wild-type protein. Therefore it is important to test whether the fusion protein is fully functional and to engineer the fusion proteins to their endogenous genetic locus, replacing the wild-type allele, so that the dosage and timing of the expression is the same as the wild-type protein. Furthermore, newly translated GFP proteins are not fluorescent within the first ~1 h, and they tend to dimerize. Monomeric variants of GFP and variants with shorter folding-maturation times should be chosen when required [19].

Methods for visualization of bacterial chromosome in living cells

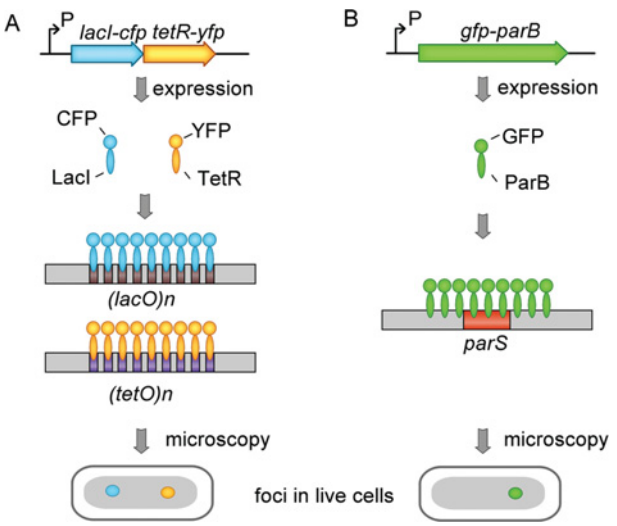
So far, there is no method to visualize specific chromosomal DNA regions directly. Instead, methods at present utilize fluorescently tagged DNA-binding proteins that recognize their cognate binding sequences in the chromosome. Examples are the FROS (fluorescent-repressor-operator system) and ParB–*parS* systems [25] (Figure 2).

FROS was the first technique developed to visualize specific genetic loci in living cells (Figure 2A) [26,27]. It was first applied to eukaryotes with tandem copies of *lacO* and *tetO* arrays inserted into specific chromosome regions. GFP variants with different emission spectra are tagged to LacI and TetR and are expressed in the cell. The specific binding of the repressors to their cognate operators allows the visualization of the genetic loci by fluorescence microscopy. This system was further developed to make genetically more stable arrays [28]. Conventionally, ~240 copies of the operators are used. With the development of more sensitive cameras and brighter fluorescent proteins, an array with 64 copies of operators or even less is enough for visualization [29]. This system allows the simultaneous visualization of two (and potentially more) genetic loci.

The specific binding of P1 plasmid partitioning protein ParB to its recognition sequence *parS* has also been used to label chromosome regions in living cells (Figure 2B) [30]. A GFP fusion of ParB is expressed in a cell with *parS* inserted into a locus of interest. The fluorescent ParB proteins are loaded on to the *parS* site and spread out to adjacent DNA sequences up to several kilobase-pairs. The localization and dynamics of the locus can be followed by using fluorescence microscopy. Combinations of ParB–*parS* with different specificities allow the simultaneous tracking of two or more loci at the same time [31]. However, this system is restricted

Figure 2 | Visualizing chromosome loci in living cells

(**A**) FROS. Fluorescent-tagged LacI and TetR are expressed in a cell with *lacO* and *tetO* arrays inserted into specific regions on the chromosome. The localization of the operator arrays can be detected by fluorescence microscopy. (**B**) The ParB–*parS* system. The *parS* sequence is inserted into the chromosome. GFP-tagged ParB protein is expressed. GFP–ParB proteins are loaded on to parS site and spread out to adjacent DNA sequences. The localization of the genetic locus is followed by fluorescence microscopy.



to *Escherichia coli*, which does not possess a partitioning system.

Drawbacks with both FROS and ParB–*parS* systems include the requirement of the introduction of exogenous DNA sequences into the genome, and that overexpression of LacI and TetR repressors or ParB protein can interfere with the replication and segregation of the chromosome. Care should be taken to ensure either low levels of expression or the addition of the appropriate inducers in the case of FROS [28,32,33]. However, when used appropriately, these systems have a better resolution than FISH.

Using these methods, it is now clear that bacterial nucleoids are not the aggregates of randomly-coiled DNA that people used to believe, but are very well organized with a specific chromosome region localized to specific cellular positions. One pioneering study using FROS to label multiple loci in *Caulobacter crescentus* revealed that the genetic map is recapitulated in the cell [34]. The replication origin is localized at one pole and the terminus at the other, with markers distributed linearly in between according to the genetic map. In slow-growing *E. coli*, using both FROS and ParB–*parS* systems, it has been revealed that the origin is located in the mid-cell and the left and right replication arms are located on either side of the origin [33,35]. The organization of the chromosome also resembles the map position with loci distributed linearly from the origin to the poles and the terminus region spanning one pole to the other linking the two replichores. Following the cell growth by time-lapse microscopy, it is intriguing to see that this

left–right organization is usually preserved in the next generation with most cells adopting left–right–left–right organization rather than a more intuitive bilateral symmetry [33].

As mentioned earlier, overexpression of the repressors can cause replication blockage. In most circumstances, this is a problem to be avoided. However, if required, this system can be utilized as a controllable, site-specific road-block for DNA replication [36], which is a powerful tool for investigations of replication stalling and restart at different loci and in different situations.

Fluorescence microscopy in bacteria

Typical bacterial cells studied in laboratories are 2–4 μm long and 0.5–1 μm in diameter. They can be visualized by both upright and inverted microscopes with $\times$100 objectives. Traditionally, wide-field epifluorescence is used. Snapshot microscopy can be used for fixed cells and living cells and a large sample size can be collected for analysis. Combining deconvolution techniques or confocal microscopy, it is possible to reconstruct the three-dimensional architecture of proteins in both fixed and live samples [37].

In order to follow cellular motions of living samples over time, time-lapse microscopy can be performed: successive frames separated by a certain time interval are taken. Cells are grown on a slide mounted with an agarose medium. The environment is temperature-controlled, using an air chamber or heating block for the slide. Indeed, it is the combination of snapshot and time lapses that gives the most informative data. Using systems now available, visualization of the dynamics of one single protein over time in a living cell is not a dream [38].

Future perspectives

Despite the advances during the last decade, plenty of questions remain unanswered in bacterial cell biology. Imaging techniques will help to sharpen the current picture of the organization in the bacterial cell and nucleoid, along with the revelation of the mechanisms involved in establishing and maintaining this level of organization. Further, these techniques will contribute to the study of a variety of biochemical processes in the context of a living cell, ideally at the level of single molecules. Even after their long relation with the study of molecular biology, bacteria continue to be both a subject of study and amenable model organisms.

The current tools and techniques to visualize molecules in the cell have strong limitations, the overcoming of which will hasten the pace in the field. Perhaps the biggest of these limitations are in the fluorescent tags and in the spatial resolution of the current systems; for both issues, alternatives are being developed [10,39–42]. The capability of incorporating synthetic amino acids in living cells will hopefully lead to the generation of small and efficient fluorophores covalently linked to proteins inside the cells [43].

Developments in microscopy will hopefully be accompanied by a parallel improvement in the ability to manipulate the physiology of cells under the microscope in real time. Perturbing the normal situation, using chemical inhibitors and mutants, will help in revealing the mechanisms of chromosome dynamics and the assembly, action and disassembly of molecular machines in time and space. The effect of chemicals on cells is usually very rapid and can be followed over time. However, chemicals that affect defined pathways are not always available and indeed relatively few protein-specific inhibitors are known. Although temperature-sensitive mutants provide one route to switch off protein function, the inactive proteins are still present at restrictive temperature and it is often unclear what effects the temperature-shift has on the protein. In an ideal world, one would like to remove a specific protein function instantly, for example, by rapid direct protein cleavage using TEV (tobacco etch virus) protease [44] or targeted destruction using the SsrA tag [45], to study phenomena with 'real-time genetics'.

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