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Highlights

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Single-molecule super-resolution imaging in bacteria

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▶ Single-molecule super-resolution imaging methods (PALM, STORM, PAINT). ▶ Imaging bacterial ultrastructures by single-molecule super-resolution microscopy. ▶ Super-resolution imaging: advantages, limitations and perspectives.

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Current Opinion in Microbiology

Single-molecule super-resolution imaging in bacteria

DI Cattoni[1], JB Fiche[1] and M Nollmann

Bacteria have evolved complex, multi-component cellular machineries to carry out fundamental cellular processes such as cell division/separation, locomotion, protein secretion, DNA transcription/replication, or conjugation/competence. Diffraction of light has so far restricted the use of conventional fluorescence microscopy to reveal the composition, internal architecture and dynamics of these important machineries. This review describes some of the more recent advances on single-molecule super-resolution microscopy methods applied to bacteria and highlights their application to chemotaxis, cell division, DNA segregation, and DNA transcription machineries. Finally, we discuss some of the lessons learned from this approach, and future perspectives.

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Introduction

Fluorescence microscopy has proven to be a powerful tool in biology, and medical sciences. The use of visible light as a probe enables non-invasive observation of protein organization and localization in live cells with high specificity. However, the maximum resolution attainable in standard fluorescence microscopy is intrinsically limited by the diffraction limit of light and is several orders of magnitude lower than for X-ray or electron tomography.

This limitation is considerably acute for bacteria, as 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 important bacterial machineries, often smaller that the diffraction limit, could not be directly probed *in vivo*. Recently, a number of new microscopy methods that beat this diffraction barrier have been developed (termed super-resolution microscopy or SRM, and reviewed in [1•,2]). Structured illumination microscopy (SIM) can be easily used with typical immunofluorescence labeling protocols and dyes but is limited by diffraction to ~100 nm lateral and ~300 nm axial resolution (depending on emission wavelength). Stimulated emission depletion microscopy (STED) truly breaks the diffraction barrier, but the resolution achievable in practice is limited by the photo-physics of the fluorophores and the high intensities of the depletion beam.

In this review, we will concentrate on SRM methods relying on the detection of single molecules (hereafter smSRM [3–5]) as they provide the highest resolution, are applicable to live cells, and have been the most prolific and successful when applied to bacteria. We will describe recent developments in smSRM, report on applications of smSRM in bacteria, discuss the limitations of these methods, and the directions in which new developments may provide new insights to the field of microbiology.

Single-molecule super-resolution microscopy

In conventional microscopy, the fluorescence signal emitted by individual fluorophores located at distances smaller than the diffraction limit of light overlap in the image plane to form a diffraction-limited spot, in which dynamic and higher-order structural information is lost (Figure 1A). To beat the diffraction barrier, smSRM methods take advantage of two key ingredients: (1) the stochastic photo-activation of a single fluorophore per diffraction-limited volume at any given time, and (2) the localization of single fluorophores with nanometer spatial resolution (Figure 1B, upper panel). These two steps are sequentially repeated until all emitters are localized, and from the superposition of the single-molecule positions the structure of the molecular complex or ultra-structure is reconstructed at super-resolution (Figure 1B, lower panel).

Several smSRM strategies have been designed, and they only differ on the fluorescent probe used and the mechanism for achieving stochastic photo-activation. Traditionally, the use of photo-transformable fluorescent proteins (PTFPs) has been attributed to photo-activated localization microscopy (PALM/fPALM) [3,4], while the use of organic dyes has been attributed to stochastic optical reconstruction microscopy (STORM) [5,6]. Stochastic photo-activation can be achieved by PTFPs that switch between either two well separated fluorescent states (with different spectral characteristics) or a fluorescent state and a non-fluorescent dark state. Several groups have also demonstrated that standard fluorescent proteins such as eYFP [7,8] can be turned into a

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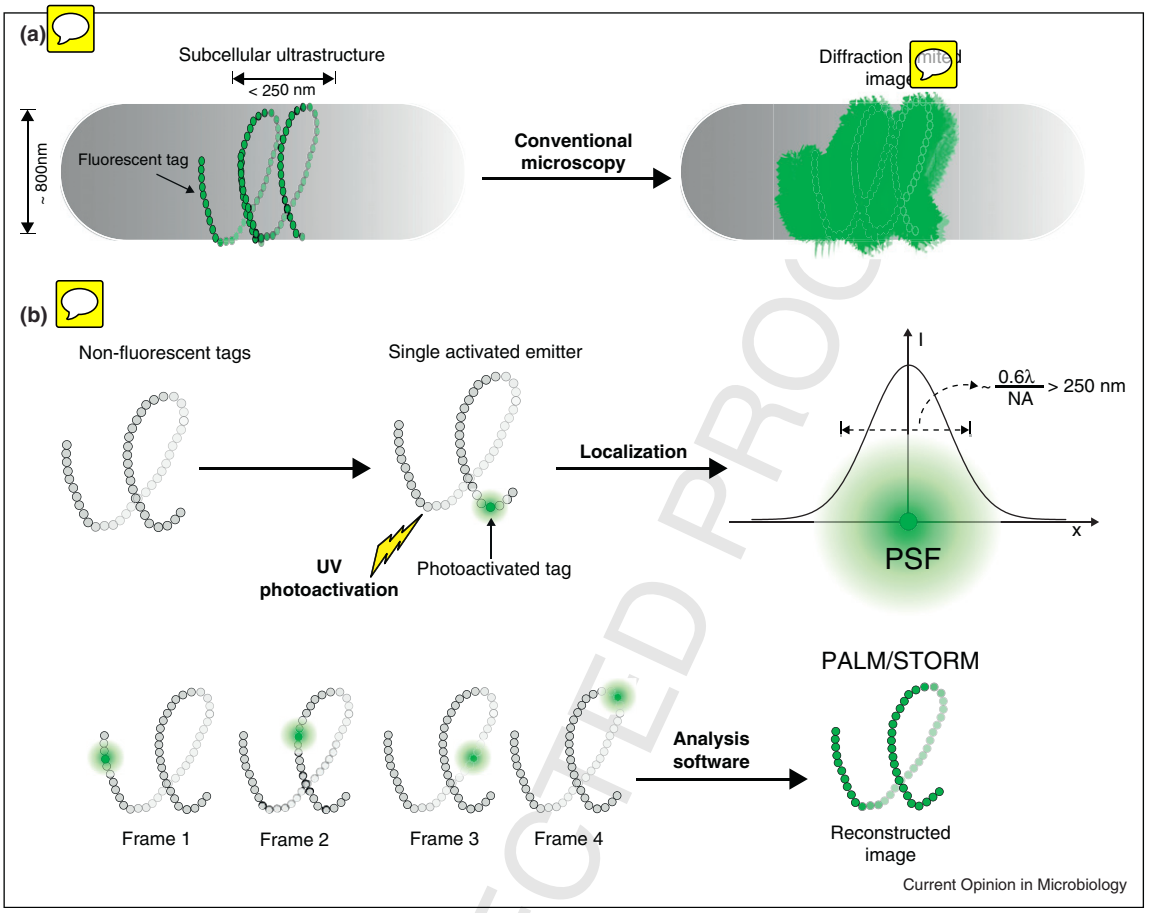
Figure 1



Principles of single-molecule super-resolution microscopy. **(A)** In a conventional microscopy image of a subcellular structure with one of its dimensions smaller than the diffraction limit (~250 nm), all individual fluorophores emit simultaneously and internal structural or dynamical information cannot be resolved. **(B)** PALM/STORM uses photo-switchable or photo-activatable fluorophores. Initially, these fluorophores are in a non-fluorescent state and irradiation with light of the appropriate wavelength induces the stochastic photo-activation of a single fluorophore per diffraction-limited spot. Image analysis is used to automatically detect the fluorophore and its mean position is determined by PSF fitting (upper panel). Sequential cycles of photo-activation/localization/photobleaching are used to obtain the localization of each fluorophore in the structure. These coordinates are finally combined to reconstruct the structure at a resolution that depends on the labeling density and the localization precision with which single emitters were localized (lower panel).

non-fluorescent form and be reactivated. PTFP probes are ideally suited for *in vivo* smSRM studies. However, multi-color imaging remains challenging given the limited diversity of bright and stable PTFPs emitting at wavelengths above 600 nm [9,10].

Alternatively, stochastic photo-activation can be achieved by conventional organic dyes coupled with specific buffer conditions that promote their entry into a stable, non-fluorescent dark state. The stochastic return to a fluorescent state can be modulated by a second (photo-activation) laser and buffer conditions [11,12]. Multi-color imaging is possible with STORM [13] thanks to the wide variety of organic dyes available [11,14], but the specific buffer conditions traditionally required cell fixation. However, it was recently shown that live-cell imaging was possible when using either thiolated compounds naturally found in cells [15] or by strategies based on SNAP-tag labeling [16].

At last, point accumulation for imaging in nanoscale topography (PAINT) [17] relies on the stochastic and transient binding of fluorescent probes to the structure of interest. Binding of each single probe that hits and binds the object produces an increase in fluorescence emission that can be used to localize the probe with high-precision. The accumulation of individual binding and detection events is then used to obtain a super-resolution image. This powerful method can be used to monitor the cell surface [18•] as well as for imaging the dynamics of membrane biomolecules in living cells [19].

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The second key step required for smSRM is the ability to localize single emitters with nanometer precision. The image of a single emitter in the sample plane on a camera is best described by the point-spread function (PSF) of the microscope. By fitting a two-dimensional Gaussian distribution to the experimental PSF (Figure 1B, upper panel), single molecules can be localized with nanometer precision [20]. The maximum localization precision essentially depends on the signal-to-noise ratio, pixel size, and background noise [21], and typically reaches ~30 nm. Other, often more efficient methods have been developed to localize the center of single emitters [22–28], but care must be taken as they often compromise localization precision for speed [29]. Finally, a super-resolution image is obtained by combining the coordinates and localization precision of each single emitter in the sample plane (Figure 1B, lower panel). The resolution of the final image will be affected by the localization precision attained, by the density of emitters (Nyquist criterium), by the ratio between on and off states, by the ability to correct lateral and axial drift, and by the distance between the protein and the fluorophore [14,30,31]. This procedure, however, only provides high-resolution information in two dimensions. To extend smSRM to 3D, several methods have been developed, including astigmatism [32], multiplane [33], double-helix point-spread function microscopy [34], and interferometry [35]. Typically, these methods achieve ~50 nm resolution in the axial and ~30 nm in the lateral direction, their main current limitation being the depth of field (typically ~500–1000 nm).

Imaging of subcellular ultrastructures in bacteria

Several studies have profited from the high labeling specificity and high resolution obtained with smSRM to solve the architecture and composition of complex intracellular structures in bacteria. One of the earliest examples of how super resolution can access structural information otherwise unavailable by other methods was the study of chemotaxis in *Escherichia coli* (*E. coli*) [36••]. In this study, smSRM was used to map the cellular locations of three proteins central to bacterial chemotaxis (Tar, CheY, and CheW) by using mEos2 fusion tags with a precision of 15 nm. Conventional microscopy shows that these proteins assemble in two clusters at the cell poles (Figure 2A, left). The resolution increase obtained with PALM imaging unveiled unprecedented detail in the internal structure and composition of these clusters. These data allowed for the determination of the distribution of chemoreceptor cluster sizes, which was shown to decay exponentially with no characteristic cluster size (Figure 2A, right). These results support the notion that clusters stochastically self-assemble without direct cytoskeletal involvement or active transport.

One of the most abundant and perhaps most important proteins for prokaryotic chromosome organization are nucleoid associated proteins (NAP). A recent work by Wang *et al.* [37•] used smSRM combined with a chromosome-conformation capture assay (3C) to study the distribution and structure of major NAPs (H-NS, HU, Fis, IHF, and StpA) in *E. coli*. Here, smSRM was used to characterize the number of NAP clusters per chromosome, their size and distribution in different growth conditions. In this study, co-localization of super-resolution images of H-NS-mEos2 and eYFP-labeled gene loci were used to show that H-NS-regulated operons are preferentially sequestered into these clusters (Figure 2B). The combination of super-resolution with 3C is rather appealing since it proved that the structural features observed by smSRM were the consequence of long-range chromosome interactions induced by H-NS clustering. Similarly, by using the blinking and photoswitching properties of eYFP, Lee *et al.* [38] were able to reveal in *Caulobacter crescentus* (*C. crescentus*) the formation of small clusters for another NAP (HU) that vary their distribution accordingly to cell cycle, suggesting a key role in bacterial chromosome organization.

Recently, smSRM was used to image and characterize subcellular bacterial machineries involved in the regulation of cell shape, growth, division and plasmid segregation. First, investigation of *C. crescentus* MreB, a cytoskeletal actin homolog involved in the determination of cell shape, by time-lapse smSRM suggested that filaments acquire a helical arrangement in stalked cells while they form a midplane ring in predivisional cells [8]. Interestingly, more recent studies showed by total internal reflection fluorescence microscopy (TIRF) that *Bacillus subtilis* MreB forms discrete patches that move circumferentially around the long axis of the cell [39,40]. Further smSRM studies on MreB may, in future, reveal the architecture, dynamics and function of MreB filaments in different bacteria. Second, Fu *et al.* [41] used smSRM to image the FtsZ filaments responsible for the constriction of the division septum in live and fixed *E. coli* cells. These studies on filamenting proteins illustrate the difficulties encountered when using smSRM to image dynamic filaments, such as poor labeling density and possible artifacts induced by PTFP tagging. Third, the use of smSRM allowed to further clarify the architecture and mechanism of chromosome segregation in bacteria by the mitotic-like Par system. By using two-color super-resolution co-localization of ParA and ParB in *C. crescentus*, Ptacin *et al.* [42] were able to show that conventional microscopy images of retracting clouds of ParA moving away from the ParB-parS centromeric-like structure indeed corresponded to a narrow linear structure (~40 nm wide) oriented along the long axis of the cell. Finally, a recent study used a combination of PAINT and 3D-smSRM [18•] to obtain two-color 3D images of the

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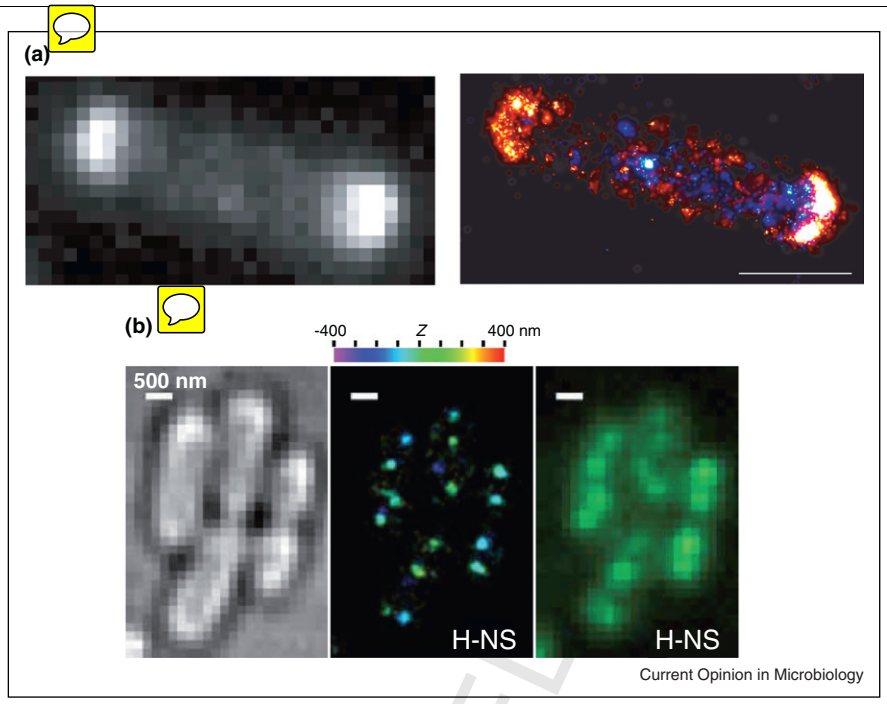
Figure 2



PALM analysis of the *E. coli* chemotaxis network and distribution of the nucleoid associated protein H-NS. **(A)** Diffraction-limited epi-fluorescence image of trans-membrane chemoreceptor Tar (left). Superposition of PALM images using TIRF (blue-white spots) and epi-illumination (yellow-orange spots) of mEos2-labeled Tar (right). Each individual emitter is represented as a 2-D Gaussian distribution whose width is the positional error for that protein. Scale bars indicate 1 μm. **(B)** Bright field image of *E. coli* cells (left) and conventional epi-fluorescence image of the nucleoid-associated protein H-NS (right). 3D subdiffraction imaging of mEos2-labeled H-NS (middle). The *z* coordinate of each localization in the 3D STORM image is color-coded according to the color bar (top).
Figures reproduced from [36••] and with permission from [37•].

cytoskeletal shape-determinant protein crescentin and the cell surface in *C. crescentus*.

Conclusions and perspectives

Bacteria have evolved complex, multi-component cellular machines to execute key processes in bacterial metabolism, such as the cell division/separation apparatus, the flagellar motor, the FoF1 synthase, protein secretion machineries, or conjugation/competence apparatus. Perhaps because of the relatively small size of bacteria, the typical dimensions of these machines are of the order of tens to hundreds of nanometers. Diffraction of light has so far limited the use of conventional fluorescence microscopy to reveal the composition, internal architecture and dynamics of these important machines. smSRM partly overcomes these limitations and has been already used to address some of these questions. It is important, however, to realize the practical limitations and caveats of smSRM.

First, a large density of single emitters need to be detected to reach high-resolution in the Nyquist sense, requiring long acquisition times (seconds to minutes). The intrinsic photo-activation, and intermittency timescales of the probe, as well as its brightness and contrast can also considerably limit the maximum speed at which smSRM images can be acquired and the maximum localization precision that can be attained. As a consequence, complexes or structures with dynamics faster than the imaging time cannot be imaged by traditional smSRM. In some cases, however, it is still possible to obtain dynamical information by single-particle tracking PALM (sptPALM) [43] or time-lapse PALM [8]. In the latter, when structural dynamics are slow with respect to PALM acquisition times, one can perform intermediate reconstructions at fixed time intervals to obtain a time-lapse PALM imaging series. Second, live multi-color co-localization is currently limited caused by the lack of bright, high-contrast and stable PTFPs with non-overlapping fluorescence emission spectra. Different strategies are currently being employed to counteract these drawbacks: (1) rational design of novel or improved photoactivatable/photoswitchable proteins [44–46], (2) design of chemical tags that can be used to label intracellular proteins with organic dyes in live cells [15,47,48], (3) new smSRM methods have very recently been developed that use traditional, non-photoactivatable GFP variants and could provide additional choices to achieve multi-color super-resolution imaging [49,50•]. Third, smSRM experiments in live cells are intrinsically low-throughput. Future advances in high-throughput automatized smSRM should

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give access to statistical measures key to address structural and dynamic heterogeneities (at nanometer and second scales) in populations at the single cell level. Coupling of high-throughput smSRM with algorithms providing real-time detection of individual bacteria classified by their morphology [51,52] may be needed to investigate highly heterogeneous structures, or structures that vary over the cell cycle.

In the coming years smSRM may become mainstream, but a multidisciplinary approach involving microbiologists and biophysicists will be required to guarantee accurate data acquisition and its proper interpretation. smSRM relies on relatively simple concepts, however, several considerations must be taken into account to perform reliable smSRM experiments. Fluorophore labeling density and specificity must be carefully controlled in order to ensure that the sample preserves its native physiological state and that the average distance between emitters is smaller than the typical spatial frequency of the sample. smSRM requires long imaging times (often minutes), during which sample movement in the nanometer scale can considerably compromise image reconstruction if not taken properly into account. Whereas sample drift can be well corrected by the use of fiducial marks (i.e. fluorescent or gold beads fixed to the coverslip) and an appropriate autofocus system, movement of the cell with respect to the surface requires more careful consideration. Perhaps, fast freezing methods adapted from those pioneered for electron/X-ray tomography will be required to achieve ideal sample stability and low structural perturbation. Finally, optimal activation/excitation intensities are required to obtain high, homogeneous photon counts and a single activated emitter per diffraction limited spot in order to avoid reconstruction artifacts. A more thorough description of the current limitations and proper use of smSRM methods can be found in McEvoy *et al.* [53••].

These are exciting times for bacterial cell biology, as several rapidly-developing advanced fluorescence microscopy methods can now provide structural information at the nanoscale (smSRM, SIM, STED), dynamic information at the ms scale (TIRF; sptPALM), absolute protein counting (Number and Brightness analysis; single-molecule microscopy), and direct quantification of protein–protein interactions (Fluorescence Cross Correlation Spectroscopy; Fluorescence Lifetime Imaging Microscopy). Ultimately, the integration of information obtained from these complementary techniques, together with genetics and biochemical approaches will provide the most complete picture of bacterial cellular functions and probably lead to the most exciting discoveries.

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