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# Methods adapted for visualization of bacterial divisome structure and protein-protein interactions

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## Abstract

Visualization of dynamic protein structures in live cells is important to recognize the pathways regulating biological processes. This review offers a concrete introduction to various approaches such as cryo-ET, bimolecular fluorescence complement (BiFC), photoactivatable localization (PALM), bioluminescence resonance energy transfer (BRET), structured illumination microscopy (SIM), FRET, and other techniques from the perspective of microbiological research. Super-resolution approaches are especially powerful and ideal for discovering details of small sizes of bacterial cells, which are not solvable by using traditional fluorescence light microscope. The procedure involved behind the applications of all these methods and their current use in microbiology have described here. The objective of this review is to guide researchers to pick out a suitable approach for their microbiological systems. Recent development and more precision of super resolution imaging techniques have widened our knowledge about the bacterial cytoskeleton, cell division proteins and their localization to divisome. The combination of super resolution microscopy techniques with genetic and biochemical methods would more explain the divisome role that divides bacterial cells and explore the protein-protein interactions that govern this mechanism.

## Introduction

Visualization of dynamic protein structures in live cells is important to recognize the pathways regulating biological processes. Biological processes are very complex and comprise for instance, the interaction between biomacromolecules [1], their distribution in cells [2], their structure [3] and dynamics [4]. The emergence of super resolution cell imaging techniques created unprecedented opportunities for inquiring how biological processes are carried out in their indigenous environments. Currently, with a number of imaging techniques accessible to the scientists, the function of biological macromolecules in live cells can be studied immensely. Using such techniques can show the spatial arrangement and organization of the physiological mechanism of molecules in a biological specimen. The pick of the imaging method should consider both the biological problem and the characteristics of the specimen that is examined. Bacterial cells are mostly smaller than eukaryotic cells in order of magnitude and thus raise numerous hurdles for cellular imaging. Exploring biological mechanisms within bacteria usually need high spatial resolution, as they generally take place on a much smaller scale (< 1 μm) [5]. The interactions among proteins are fundamental to very important processes of the cell, like gene expression, signal transduction, translocation of proteins, and cell cycle progression. The complexes in which cellular proteome is distributed are stable and big, maintained by an abundance of interactions among proteins. The depiction of these protein-protein interactions along with their complex assembly and disassembly in the cell is a crucial requirement to cognize cellular processes, and their failure to function in a condition of disease [6].

Strong approaches for *in vivo* cellular analysis of protein-protein interactions (PPIs) have been established, such as BiFC, bacterial two-hybrid assays, BRET and FRET [7]. The expression of targets of interest fused to fluorescent proteins (FPs) is among one of the labeling methods used to image indirectly or directly proteins with diffraction-limited and super-resolution microscopy (SRM). These SRM methods include SIM [8], DNA points accumulation for imaging in nanoscale topography (DNA-PAINT) [9], stimulated emission depletion (STED) microscopy [10], stochastic optical reconstruction (STORM) [11, 12] and photoactivatable localization (PALM) microscopies [13]. Although imaging of targets in bacterial cells has often used genetically encoded fluorescent proteins for direct visualization, indirect visualization of FPs utilizing binders with organic dyes may result in increased functionality and higher spatial resolution because of the frequently superior photophysical properties. Nevertheless, due to the limited permeability of the cell wall, it becomes difficult to gain a high labeling efficiency of intracellular proteins in the latter method [14]. The direct visualization of FPs, expressed as fusion proteins in bacterial targets, has been largely adapted in super-resolution light microscopies [15, 16]. The significant advantage of this approach is that without intensive sample preparation, bacterial samples can be directly imaged — even live. Investigators identified protein assemblies like cell division machinery [17-19], membrane microdomains [20, 21], and cytoskeleton [22, 23] in different bacterial organisms e.g. *S. aureus*, *B. subtilis*, *E. coli*, and *C. crescentus* by utilizing direct visualization of FPs with SRM. Moreover, new progress of dual-color imaging utilizing FPs for SIM [24] and STED [25] have helped scientists to achieve biological insight into the relationship between the ultrastructure of protein assemblies and their role, that would otherwise be inaccessible.

Studies had shown cytoskeleton to exist only in eukaryotic cells until the last decade. Nonetheless, bacterial FtsZ tubulin homolog, actin homolog and intermediate filaments homolog are now reported to be present [26]. Thus, these proteins have crucial importance in explaining the emergence of cytokinesis and cytoskeleton in earliest life forms [27]. In bacterial cell division, FtsZ localize to midcell site and form the Z-ring [28]. Using confocal and wide field microscopy for the visualization of localization of FtsZ proteins within the cell, being labeled with fluorescent tags, has demonstrated to be a tremendously worthful method for visualizing Z-rings in bacteria [29] and the dynamics of these Z-rings are revealed through conventional fluorescence microscopy in live *E. coli* cells (**Fig. 1**) [30].

***Immunofluorescence microscopy (IFM)***

Biological scientists used immunofluorescence microscopy (IFM) for *Bacillus subtilis*. In order to enable antibodies to enter the cell, cells were fixed and then permeabilized with lysozyme before immunolocalization of proteins with fluorescence [31]. With the use of this method, researchers observed that the membrane protein

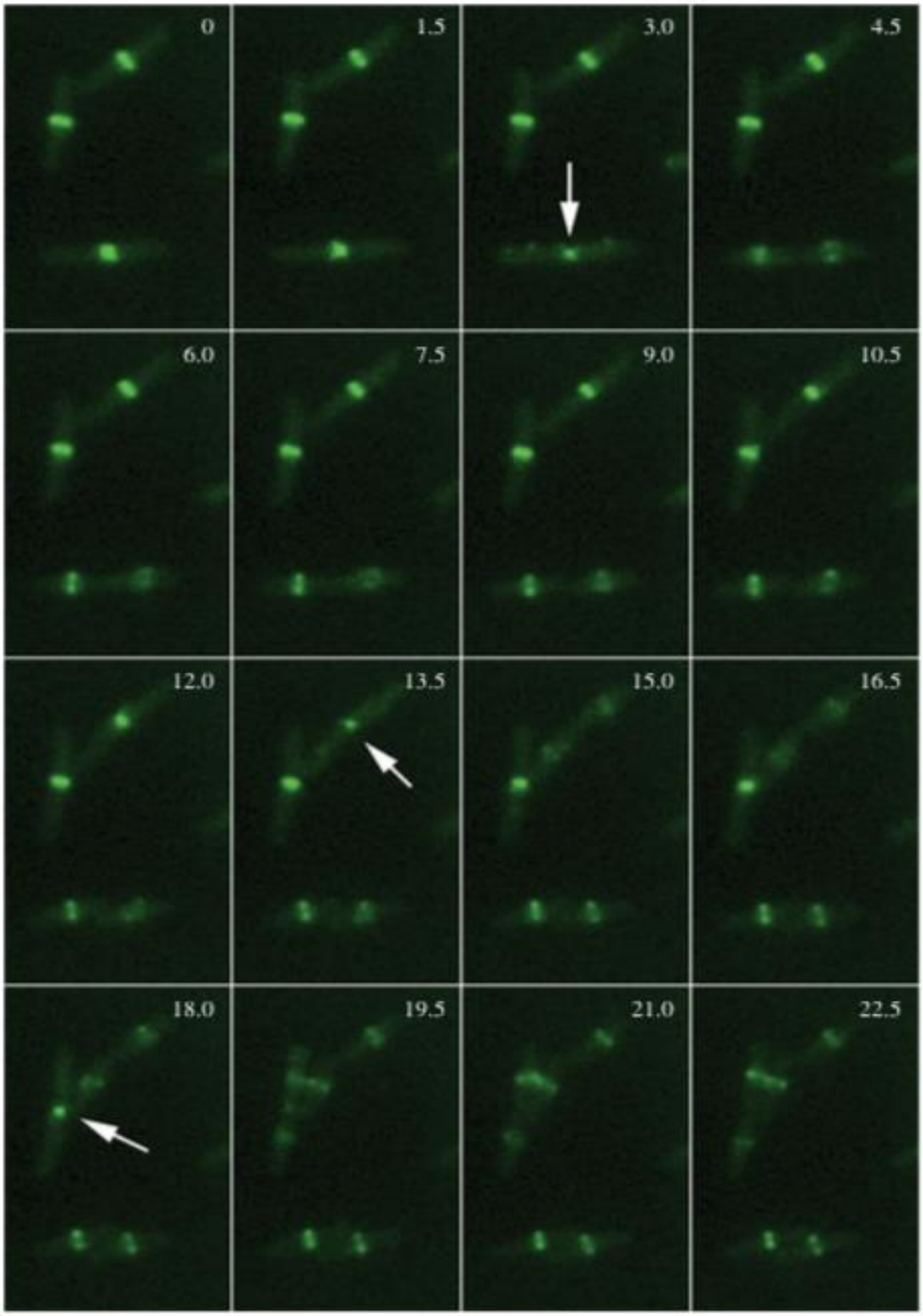


**Fig. 1:** Dynamics of FtsZ in dividing *Escherichia coli* cells. A time lapse of FtsZ-GFP production is shown in wild type cells and the observation of its localization under the microscope. Time is shown in minutes. The arrows are pointing at the constriction of the Z-rings in three cells before the septum is completed and cell is separated. FtsZ-GFP fluorescence is quickly localized again to the future cell division sites. (The figure is adapted from reference [30], and copyrights of the Royal Society).

sporulation phosphatase SpoIIE is located in the asymmetric septum, which isolates the *B. subtilis* cell from the developing spore [32]. This method was then rapidly used in *E. coli* and other bacteria in order to ensure the localization of FtsZ at midcell division sites between the segregated daughter nucleoids [33]. Other scientists also used this technique to show that different identified fts genes, such as FtsA, FtsI, FtsW and FtsQ are also located firmly at the sites of cell division, where FtsZ was also present [34, 35]. Combining this method with the fts mutants revealed that the localization of ftsN to the division sites also depends on the recruitment of these fts genes [36]. The first tentative interpretation of the recruitment dependency that in effect implied temporal hierarchy was rendered by both the use of cytology and genetics. Genetics alone would have been very hard to examine.

***Green fluorescent protein (GFP)***

The GFP, as a genetically encodable fluorescent tag, was discovered again around the same time as IFM was developed for bacteria [37]. As was the case for IFM, which was first used for eukaryotic cells, but a team of researchers quickly developed GFP for using in bacteria. They used it for the localization of proteins during *B. subtilis* sporulation in specific cell compartments [38]. Soon afterwards, other scientists used FtsZ–GFP fusions for the first time to depict FtsZ and FtsA in living cells [39]. Ma X, et al. recorded the first 3-dimensional image of the Z-Ring with the aid of David Ehrhardt who used another approach named as deconvolution or wide-field optical sectioning and this technique was first adapted by another group of researchers [40]. GFP tags now enabled every other protein to be localized without specific antibodies or cell fixation required. This development led to further advances that IFM alone could not achieve.

***Combined IFM and fluorescent protein tags***

The combined IFM and fluorescent protein tags explained the divisome structure in many species such as *Caulobacter crescentus, Bacillus subtilis, and Escherichia coli*. It helped scientists to explain how alike homologues are found in other species of bacteria. As a consequence of that, Z rings were discovered in a large number of different living cells, and the perturbation of these Z rings leads to impaired cytokinesis. In particular, several proteins are extremely difficult to determine with genetic or biochemical methods due to transient associations or modest phenotypes when inactivated, fluorescence microscopy has indeed been indispensable for the screening of Z ring binding proteins [41].

Various approaches are used to explain the divisome structure. One effective technique has described the successful localization of cell division proteins upon the deletion of other division proteins. Because divisome protein gene knockouts are usually harmful, these experiments are mostly carried out in model systems with highly advanced genetic tools like adjustable promoters, temperature-sensitive mutants, or suicide plasmids which can easily trigger a particular protein to be removed from the cell. Due to the ability of filamentous cells like *Escherichia coli* to stay alive for very longer time, immuno-fluorescence microscopy or Fluorescent Protein-Tagged divisome proteins were used to identify the dependency of division protein upon another. For instance, the *E. coli* cell division proteins Ftsl, FtsQ and FtsL do not require FtsN for its localization but cannot localize if FtsZ, FtsA, or FtsK are not present there [42]. This indicates that Ftsl, FtsQ and FtsL are independent of FtsN but cannot localize in the absence of FtsZ, FtsK and FtsA, which put them in the center of the recruitment dependency order. This pathway of dependence on other proteins for recruitment was almost compatible with the exact time of their noticeable assembly at the Z-ring, where the same more proteins are recruited later [29].

***Bacterial two-hybrid assays (BTH)***

The bacterial two-hybrid (BTH) variant was invented for the characterization of interactions between two proteins [43]. The proteins of interest are genetically fused to two complementary fragments, T25 and T18, which make up the catalytic domain of *B. pertussis* CyaA. Interaction between the two proteins leads to the functional complemen- tation between the two fragments of adenylate cyclase resulting in the synthesis of cAMP, which, in turn, can induce the expression of many resident genes. The proteins that interact together show blue colonies, and those with no interaction show white colonies (**Fig. 2**). Using this method, a simple genetic screening can be used to select particular clones that express a protein that interacts with a given target [44, 45].

This method has been frequently used to screen for previously undiscovered protein partners participating in different functions, such as antibiotic resistance [46], bacterial viability [47], spore wall synthesis [48] and virulence [49]. In addition, BTH has been used to identify protein interactions arising either in the cytosol or in the membrane [50]. Yet, bacterial two-hybrid analyses can give false

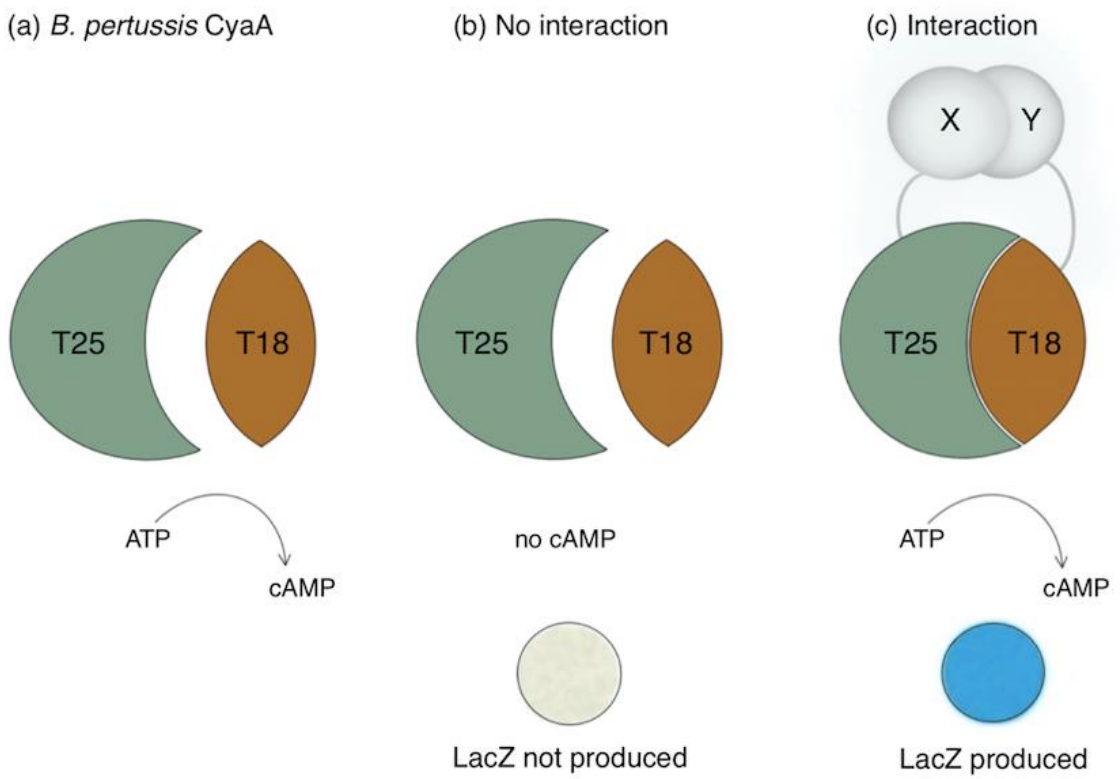


**Fig. 2:** BTH system. The B. pertussis CyaA protein consists of two functional domains, T25 and T18 (a). As the protein is split into its domains, cAMP is not synthesized and β-galactosidase (LacZ) is not produced that show white colonies (b). When the two domains are fused to two divisome proteins, X and Y, and they interact together, it causes the synthesis of cAMP, and β-glalctosidase (LacZ) is produced which show blue colonies (c).

positive and negative results and should be used primarily as a genetic screen to yield theories that can be evaluated more properly by applying other approaches.

***Bimolecular fluorescence complementation (BiFC)***

BiFC assays are frequently chosen as compared to FRET as they are simple to apply, quick to analyze, and having little sensitivity to the comparative proportions of the two proteins that interact together. In this method, the fusion of two proteins occurs with two complementary fluorescent protein (FP) fragments that are gathered as a functional reporter when there is an interaction between the two proteins. Individually, both complementary fragments are not fluorescent and high contrast is achieved regardless of the relative amount of the two interacting proteins. Nevertheless, screening protein-protein interactions (PPIs) with BiFC has raised many problems. Uncontrolled self-assembly can create ambiguous fluorescence background. In addition, In the case of BiFC established on GFP proteins family, complementation is superseded by the maturation of chromophore, resulting in the formation of permanent complex [51], whereas for BiFC established on phytochrome-based infrared FPs, biliverdin chromophore binding is sluggish and mostly contributes to irreversibility [52, 53]. The slow development of fluorescent complexes prohibits the observation of transitory protein-protein interactions and the output of vigorous studies comprising active and inactive states and can cause dominant negative or dominant positive results [54]. This method has been used to validate FtsZ and ZipA interactions with one another, in addition to ZapB with itself. Furthermore, this method has discovered new unpredictable interactions between ZipA-ZapA, and ZipA-ZapB [55].

***Foster resonance energy transfer (FRET)***

FRET assays focus on a non-radiative, distance dependent energy transfer from one fluorescent donor to an acceptor and enable the analysis of fluorescently labeled protein interactions in living cells [56]. The key benefits of FRET are the non-invasive, quantitative and real-time measurements of intracellular protein interactions. The assessment of transitory interactions between proteins, like those

participating in signal transduction, is therefore especially useful. Variations in protein conformation can also be tracked whenever changes occur in the distance between fluorophores linked at various areas of protein of interest, a method employed for establishing many FRET-based reporter assays [57]. Both fluorescent dyes and fluorescent proteins (FPs) can be applied for labeling [58], however, particular in-vivo fluorescent dyes labeling is not easily done in bacteria as cell membranes are less permeable and micro-injection targets are small. The method for bacteria is, therefore, the expression of fluorescent protein fusions. Many mutants of green fluorescent protein (GFP) could be applied as FRET donor/acceptor pairs, in which CFP, cyan and yellow fluorescent proteins, and YFP are most widely used [59]. FP labeling has a benefit of complete particularity. In bacteria having inducible expression systems, it restricts the level of donor and acceptor proteins in the cell. A drawback of fluorescent proteins is their comparatively large sizes, where the fluorescent center is not allowed to be near to one another, thereby reducing FRET efficiency [60].

The first application of FRET to explore interactions among proteins, a group of researchers evaluated interactions among *E. coli* cell division proteins such as FtsZ, ZapA, FtsI, FtsQ, FtsN and FtsW. They used mKO FP as a donor and mCherry as acceptor fusion tags and observed robust FRET among proteins such as FtsZ-FtsZ, FtsZ-ZapA, and ZapA-ZapA. They also discovered that other cell division proteins FtsI, FtsW, and FtsN had interactions, and FtsN has interaction with itself. Most interestingly, they assessed substantial FRET for ZapA and FtsN, and ZapA and FtsI [61].

***Combination of FRET and FLIM (FRET-FLIM)***

When combined with molecular spectroscopy or optical imaging techniques, Förster Resonance Energy Transfer (FRET) enables accurate distance measurements between nanoscale interacting molecules (< 10 nm). Fluorescence lifetime imaging microscopy (FLIM) offers a sensitive approach to measure FRET by quantifying the diminish in the lifespan of the donor fluorophore when an acceptor fluorophore is within ∼10 nm [62]. FRET-FLIM enables spatial distribution measurements of molecule ensembles in many structural states; yet, the traditional microscopy techniques used for FLIM are diffraction-limited, reducing the resolution of these measurements. Single-molecule FRET is an implanted technique to observe single protein conformation and dynamics [63]. FRET-FLIM measurements were successfully applied in combination with single-molecule microscopy. This technology can obtain a lot of information about intracellular protein-protein interactions in the cells [64-66]. FRET-FLIM and structured illumination microscopy were also combined [67].

FRET and FLIM combined together were applied in *Streptomyces* to evaluate interactions between FtsZ and two positive spatial regulators of FtsZ positioning, SsgA and SsgB [68].

***Stimulated emission-depletion (STED)***

The first far-field super-resolution method established was stimulated emission-depletion microscopy [69]. The apparatus is same like a confocal microscope where depletion laser is added that induces excited molecules in a donut-shaped region around the central confocal spot back to the ground. The molecules within a range of 30–80 nm of the excitation spot center is observed in this manner. This depletion conception has been applied to fluorophore photoswitching by the use of a donut shaped depletion beam to turn fluorophores off rather than trigger emission. This method, known as RESOLFT (Reversible Saturable Optical Fluorescence Transitions), extenuate the harmful consequence that the high-power depletion beam might have on cell viability due to the reason of using RESOLFT depletion beam at far low power than the stimulated laser depletion (100–500 MW cm−2). Only two bacterial proteins were analyzed using STED and RESOLFT due to the complex instrumentation needed. Adjacent MreB filaments in live *Escherichia coli* cells using RESOLFT, which were unsolvable by confocal microscopy, were differentiated, and the average width of these filaments was found to be 70 nm [70]. Another group of researchers found rather irregular structures of FtsZ helix spanning the length of *Bacillus subtilis* cells by the use of a commercial STED microscope (Leica TCS STED) [71], identical to those found by PALM method in the *Escherichia coli* midcell [72]. However, due to the achievement of super-resolution images without additional data processing, STED has drawn large attention, thus significantly minimizing the risk of artifacts production [73].

***FLIM-FRET/STED combination***

FLIM-FRET method simply requires identifying donor decay times and also makes it possible to differentiate between interactive and non-interactive donor fractions, a disadvantage of intensity-based FRET measurements [74]. Nevertheless, if the two

interacting proteins change their intracellular location when they are measured or both are engaged in configuration changes, the FLIM-FRET missing direct information about structural and mechanistic insights. The combination of STED microscopy with FLIM-FRET will thus overcome elaborated structural along with quantitative data regarding the molecules of interest. Combined FLIM-FRET/STED recordings have been described recently [75-77].

***3D-structured illumination microscopy (3D-SIM)***

The structures inside the cell can be visualized completely 3D by using 3D-SIM method. This is the only super-resolution microscopy that provides a 2-fold improvement in both lateral and axial resolution to produce genuine 3D super-resolution images [78]. Researchers described using 3D-SIM in two Gram-positive organisms to visualize the Z ring: rod shaped *Bacillus Subtilis* and spherical *S. aureus*. They have shown that in both organisms the general architecture of the Z-ring is quite identical and consists of a heterogeneous distribution of FtsZ, indicating an irregular Z-ring architecture. The improved axial resolution potentiality of this method only permits to visualize how FtsZ is localized to Z-ring along with the visualization of dynamic changes in live cells over time which happen to this FtsZ localization by the use of another fast-live 3D-SIM, named as OMX Blaze. The localization of other divisome proteins was also visualized to demonstrate that they have the same heterogeneous dynamic distribution at the cytokinesis site [79].

3D-SIM has many benefits over other super-resolution approaches. It can be instantly used for conventional microscopy slides. It can identify four wavelengths in the same sample while using standard fluorescent proteins, dyes, or a combination of botit. 3D optical sectioning can be effectively performed in 3D-SIM, thus improving resolution in both the lateral and axial directions. 3D-SIM has a significantly higher speed, making this method ideally suited for live cell imaging in contrast to other super-resolution techniques [80-82].

***Photoactivated localization microscopy (PALM)***

There is another type of super-resolution method, which could locate single molecules to a tiny area, was applied. These techniques, termed PALM or STORM (stochastic optical reconstruction microscopy), depend on fluorescent tags that are in the off state until they have been turned on by particular excitation light. By the use of photoswitchable fluorescent proteins like mEos fused to FtsZ for PALM of live cells, a group of researchers discovered that the width of the *Escherichia coli* Z-ring is about 110 nm, which is an irregular assembly of FtsZ filaments [83]. These findings are endorsed by a *Caulobacter* Z ring 3D-PALM research using FtsZ-Dendra fusions, stating that Z-ring is irregular in normal cells [84]. Two-dimensional photoactivated localization microscopy (PALM) analysis of the *Escherichia coli* Z ring [72] found that the Z ring is a sloppy, continuous bundle made up of various overlapping filaments.

The Xiao laboratory recently combined molecular biology methods with PALM and demonstrated that FtsZ, ZapA, ZapB and the DNA binding protein MatP construct a protein network extending from the cytoplasmic membrane into the chromosomal replication terminus with which MatP directly interacts [19].

***Cryo-electron tomography (CryoET)***

Cryo-electron tomography (cryoET) is the preferred method for pleomorphic and heterogeneous biological specimens such as intact cells, tissues, pleomorphic viruses and variable macromolecular assemblies. In cryoET, as the sample is rotated to different angles relative to the incident electron beam, a sequence of projection images from the same object are reported. The images are then combined and reproduced in order to create a 3D tomogram. This offers an unprecedented 3D volume of a single specimen. CryoET enables 3D imaging of frozen-hydrated biological specimens similar to the native state. Structural knowledge to near-atomic resolution can be acquired under optimal conditions. By giving structural information in situ, within native cellular environments, cryo-ET can eradicate the restriction of the now established method for single-particle analysis in which the structure of isolated molecular complexes are resolved at near atomic resolution [85-87]. CryoET is a multifarious method, ideal for a wide range of different specimens, from isolated protein complexes to large eukaryotic cells [87].

There are two major methods of current cryoET application, called molecular cryoET and cellular cryoET. Usually, molecular cryoET is used to examine in vitro purified ‘single-particle’ samples, which are often pleomorphic and not conformable for cryoEM SPA (single-particle image analysis) [88]. In comparison, Cellular cryoET is implemented in large pleomorphic objects like intact bacteria and eukaryotic cells. It is hard to image *E. coli* as well as other large rod shaped cells, as they are very dense, while smaller *E. coli* minicells and other species are

recently manipulated to image the assemblies of surface proteins [89].

*Caulobacter crescentus* is peculiarly appropriate for cryo-ET examination of the divisome because its cell division occurs entirely by constriction, thereby linking the two daughter cells, which is in favor of this method [90].

## Concluding remarks

The new development and more precision of super resolution imaging techniques have widened our knowledge about the bacterial cytoskeleton, cell division proteins and their localization to divisome. The combination of super resolution microscopy techniques with genetic and biochemical methods would more explain the divisome role that divides bacterial cells and explore the protein-protein interactions that govern this mechanism.

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