Bacteria

Benefits of multi-modal single-molecule localization microscopy for microbiological studies

Introduction

Microbial cells are small, in the range of a few micrometers, and the biological structures within them reach sizes down to the nanometer scale. Measurement of structural details at this scale and investigation of molecular interaction is difficult or impossible using conventional microscopy methods such as widefield, TIRF and confocal microscopy, as cellular nanostructures lie below the resolvable limit of these microscopes (~ 200 nm). Single-molecule localization microscopy (SMLM) can overcome this limitation, providing the resolution (10-40 nm) and sensitivity to probe biological phenomena at near-molecular scale. There are different strategies to realize SMLM, which differ by the labeling approach and imaging strategy employed. The use of varying labeling strategies for different types of biological markers equips researchers with increased flexibility and allows them to address a range of biological questions. This can be, for example, the extraction of protein dynamics with single-molecule live-cell imaging or the visualization of structural details with super-resolution imaging.

The Nanoimager offers simple robust application of SMLM techniques such as dSTORM (direct Stochastic Optical Reconstruction Microscopy), PALM (Photoactivated Localization Microscopy) and PAINT (Point Accumulation for Imaging in Nanoscale Topography). These techniques could be combined in the same multiplexed imaging experiment, like published previously[1,2]. Once molecules have been localized, SMLM data can be further analyzed. The CODI environment offers simple access to many state-of-the-art algorithms, e.g. for grouping localizations into clusters or patches to measure spatial statistics of their distributions or to investigate spatial correlation using colocalization analysis. The superior resolution of SMLM together with robust data analysis can give insight into structure and function of molecules in microbes.

Summary

The Nanoimager offers simple single-molecule localization of different biological markers. Utilizing multiple labeling technologies in a single imaging experiment, different targets can be super-resolved and spatially correlated:

- **PALM** allows for super-resolution imaging in fixed and live cells using genetically encoded fluorescent proteins.
- **dSTORM** achieves molecular scale resolution with a variety of different labeling methods such as enzymatic labeling, click-chemistry, or immunofluorescence.
- **PAINT** methods are based on transient labeling, allowing for multiplexed, sequential imaging with single-molecule resolution.
- **Online analysis of single-/multi-color data using CODI** allows to extract quantitative information about structural organization and distribution of labeled molecules at the nanometer scale (cluster analysis, colocalization), as well as easy results sharing with the community.

Challenge

Choosing a fluorescent labeling strategy for SMLM strongly depends on the nature of the target molecules and structures of choice. Each of the techniques mentioned above come with their benefits and drawbacks, making the technique particularly suitable for specific target molecules (e.g. proteins, nucleic acids, lipids). However, these different strategies can be combined in a single super-resolution imaging experiment to combine the benefits of different techniques. For instance, one protein marker may have a good antibody available, which can be linked to a far-red dye for dSTORM, whereas lipids in the membranes or DNA might be transiently stained with a specific dye for performing PAINT. Photoactivatable (or switchable) protein tags genetically attached to a gene of interest allow for the PALM method to be used to localize and count the copy number per cell of a protein of interest. The ability to select these methods for the same field of view, sequentially or even simultaneously, simplifies the methodology and enables easier access to novel information, in particular spatial correlation. This can be a powerful tool in the study of subcellular architecture in bacteria[3], such as bacterial nucleoids[4], whose fine structure is unresolvable without the use of super-resolution fluorescence microscopy. At the same time, structures of the bacterial membrane can also be resolved at the nanometer scale, providing exact information about cell size and shape [1,2]. In this text, we provide a couple of examples to illustrate the capabilities of multi-modal single-molecule imaging of Escherichia coli *(E.coli)* using the Nanoimager.

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Results

In this study, *E. coli* MG1655 wild type or mutant strains were cultured following similar methods used in Spahn *et al.*[2]. Dual-color PAINT was performed for imaging the entire chromosomal DNA (the so-called nucleoid) and membrane lipids (Figure 1). The novel lipid-specific dye Potomac Gold, developed by the group of Luke Lavis (see Spahn *et al.*[2]), transiently labeled the bacterial membranes, and the DNA minor-grove binding molecule, Hoechst 33342, was covalently linked to a modern, bright far red dye, Janelia Fluor® 646 (JF_{646}) to transiently label the bacterial nucleoid structure. The big advantage of the DNA-targeted PAINT labels is that it can be used on unmodified DNA, in contrast to metabolic labeling approaches. However, both strategies can be combined, e.g. in pulse-labeling experiments.

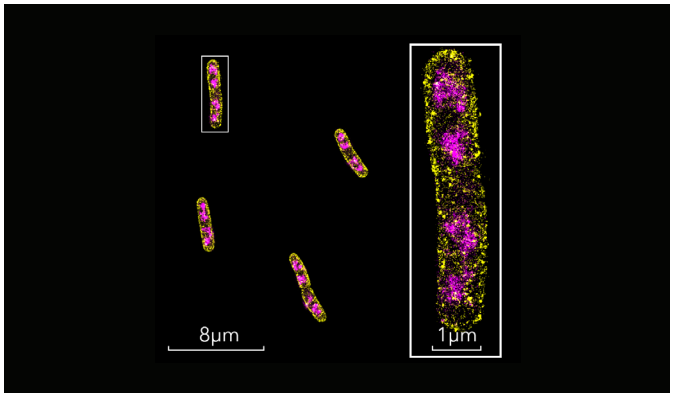

Figure 1. Two-color PAINT in fixed *E. coli* bacteria. The nucleoid was labeled with JF_{646}-Hoechst (magenta), and membrane lipids with Potomac Gold (yellow).

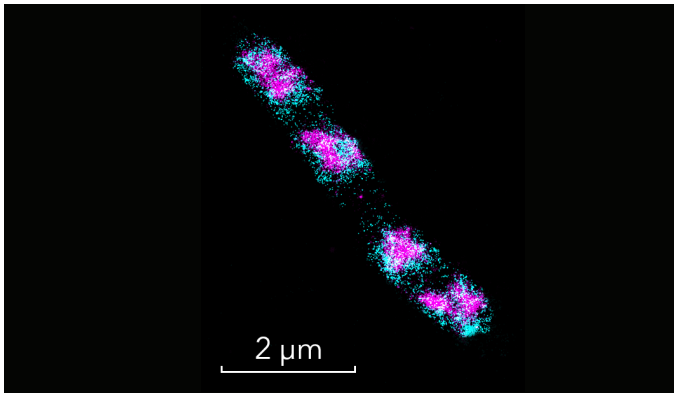

Figure 2. Two-color imaging using PALM and PAINT sequentially in the same sample. RNAP tagged to PAmCherry1 (cyan) and JF_{646}-Hoechst transiently labeling the bacterial nucleoid (magenta).

Additionally, another SMLM strategy was used to access further information of bacterial structures using mixed modalities. PALM and PAINT imaging were combined to study the spatial correlation of bacterial proteins and the nucleoid. The photoactivatable fluorescent protein PAmCherry1 was genetically fused to a bacterial RNA polymerase subunit protein (RNAP), enabling super-resolution microscopy by PALM, and with JF_{646}-Hoechst again transiently labeling the bacterial nucleoid (Figure 2). PALM enables not only determination of molecular spatial distributions, but also quantitative analysis by extracting protein copy number or performing cluster analysis[4].

As shown above, it is possible to perform multiplexed SMLM, but some considerations regarding the labeling must be considered for an optimal imaging workflow. Table 1 summarizes the characteristics of the different SMLM imaging modalities.

	dSTORM	PALM	PAINT
Best for	Very high resolution, molecular distributions in fixed cells	Protein dynamics, organization and quantification	Biomolecular organization and quantification
Labeling	Enzymatic tagging, click-labeling or immunostaining with dyes, image with blinking buffer	Genetic encoding using fluorescent proteins	Specific transient binding molecules linked to fluorescent dyes
Sample state	Fixed	Fixed and live	Fixed
Fluorophores	Organic dyes compatible with STORM blinking	Photoswitchable fluorescent proteins	Organic dyes
Brightness/Photon yield	High	Low	High
Photobleaching?	Sensitive to photobleaching	Sensitive to photobleaching	Insensitive to photobleaching
Specific buffer?	Yes	No	Optional

Table 1. Considerations for labeling optimization in SMLM techniques.

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Solution with the Nanoimager and CODI

In the past, SMLM has required the use of complex and very expensive microscope systems together with highly specialized expertise, placing such work out of the reach of most microbiologists. Further, there has been a lack of easy-to-use data analysis tools for measuring molecular clustering and distribution, morphology and numbers of molecules present.

Here we presented nanoscale imaging of structural features in bacteria, using state-of-the-art multiplex methods. These were performed on the bench, at your fingertips, on the Nanoimager. It has the power and convenience to target specific SMLM modalities, by combining dSTORM, PALM or PAINT in the same sample in one imaging sequence, on an easy-to-use desktop microscope system. Automated multi-modal, multi-color SMLM imaging was performed, using several markers of interest, labeled conveniently according to their molecular properties. The Nanoimager reveals their relative spatial distributions, providing rapid access to information that can help to understand their biological functions.

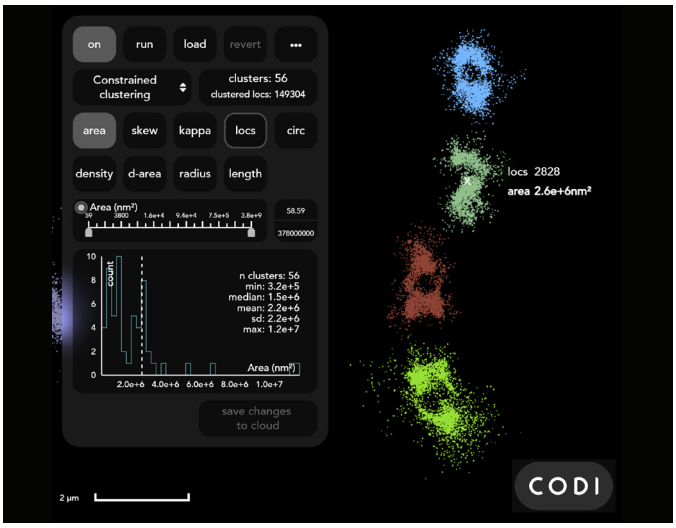

Figure 3. Object clustering analysis performed in CODI, of bacterial nucleoid marker localizations from PAINT. After object filtering, a plethora of shape and distribution parameters can easily be calculated and visualized, and shared with collaborators.

Moreover, ONI's cloud-based analysis platform, CODI, provides an easy-to-use workflow for cluster analysis, while enabling sharing and collaboration (Figure 3). The Nanoimager also includes tools for analysis and visualization of single particle tracking (SPT) experiments, to further probe molecular dynamics in live-cell imaging in multiple color channels in the same sample.

These features make the Nanoimager a unique tool to explore the subcellular organization and dynamics in bacterial studies at the nanoscale, breaking the technological barriers of super-resolution fluorescence microscopy and making it accessible to all microbiologists.

References

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