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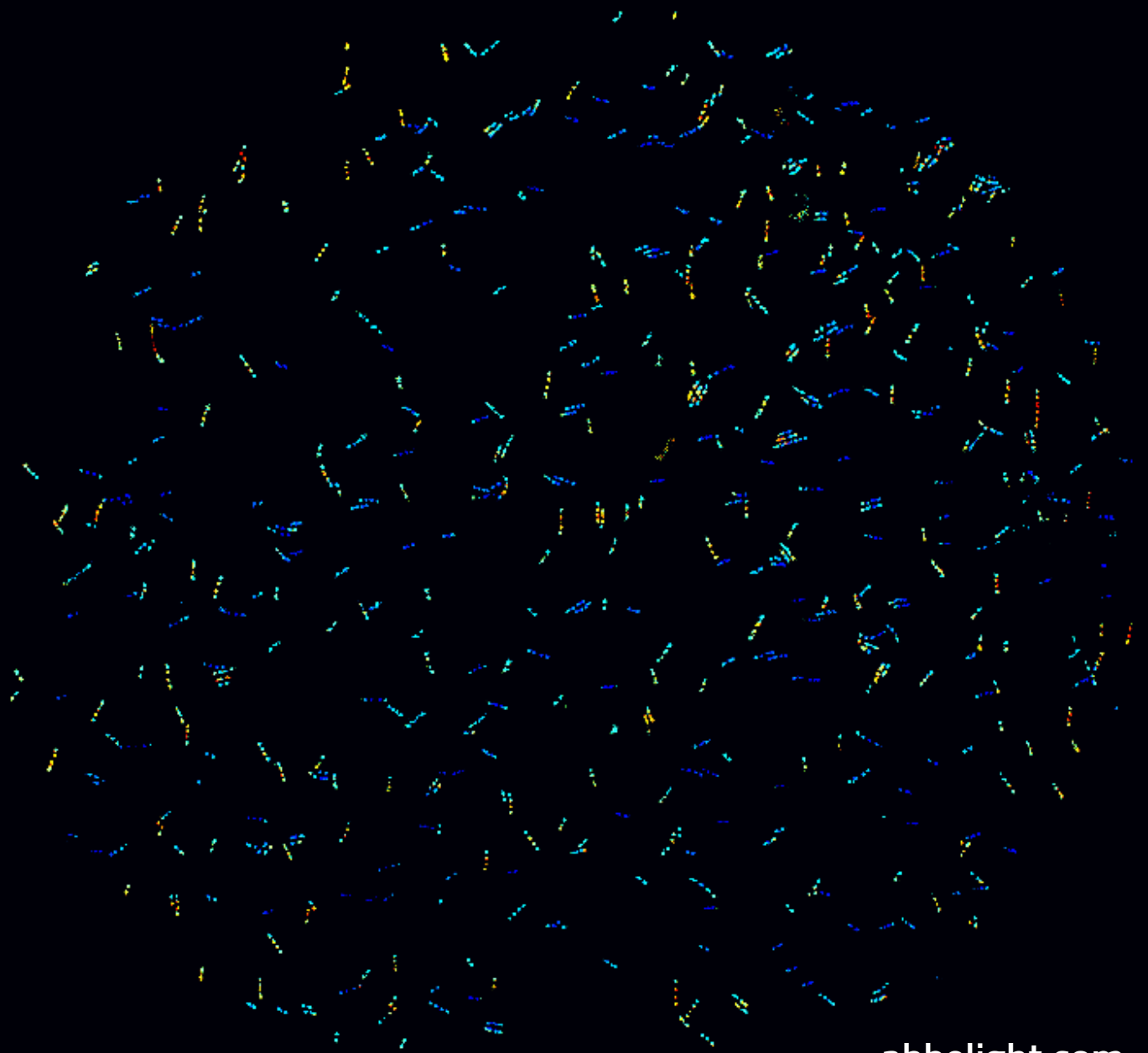
High-throughput and Quantitative Nanoscopy Imaging of Bacterial Cell Components



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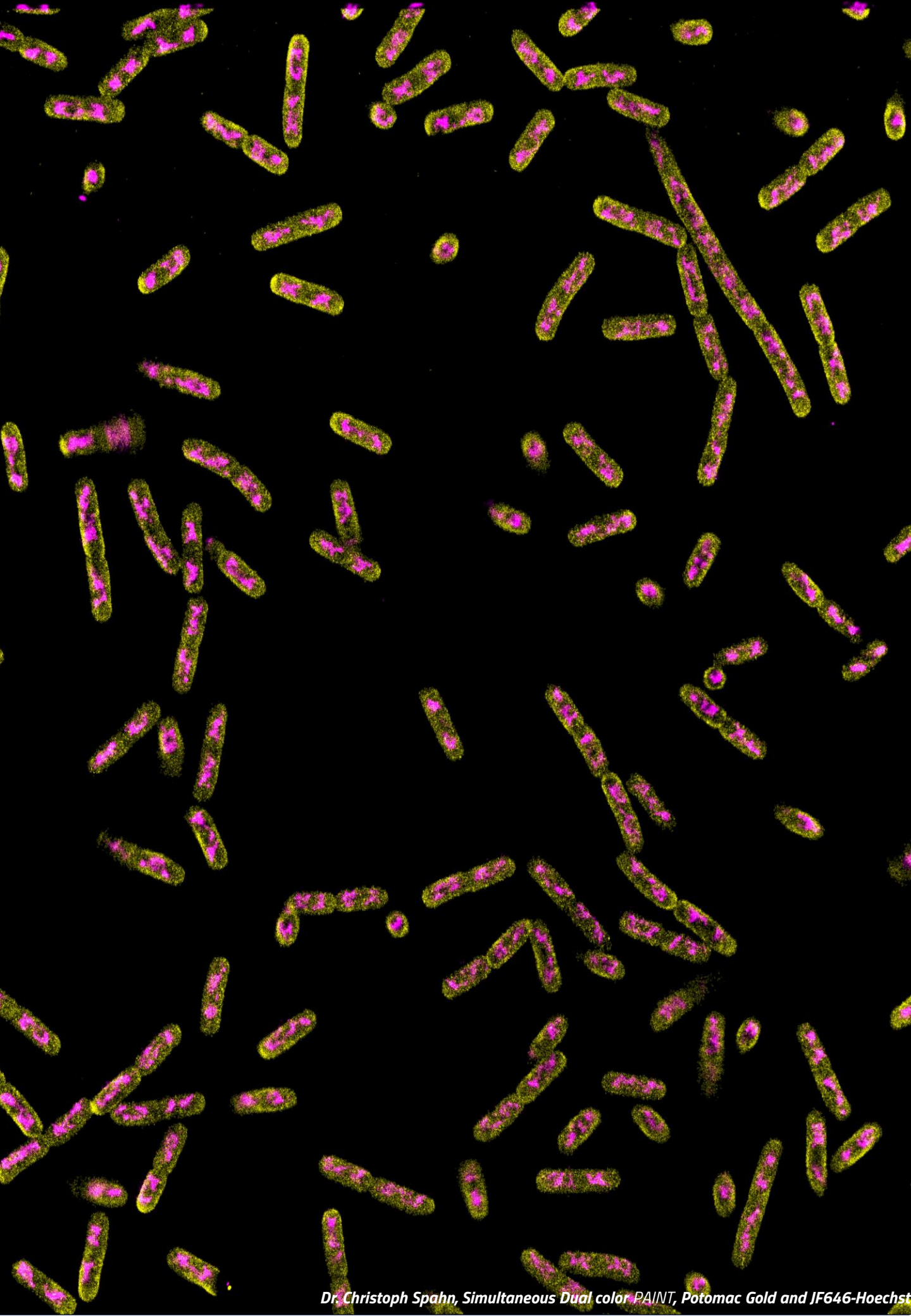

Dr. Christoph Spahn, Simultaneous Dual color PAINT, Potomac Gold and JF646-Hoechst

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High-throughput and Quantitative Nanoscopy Imaging of Bacterial Cell Components

Author: Dr. Ipek Altinoglu

Single molecule localization microscopy (SMLM) has enabled remarkable findings in microbiology. However, a Gaussian-shaped laser beam produces small and non-uniform illumination, which limits the ability to perform robust quantitative and statistical analyses of subcellular localizations from multiple cells. Abbelight's patented ASTER technology overcomes this challenge by creating a uniform and large field of illumination, which facilitates SMLM imaging and analysis of hundreds of bacterial cells in a single acquisition with fine-scale localization. Here, we demonstrate the power of ASTER for high-throughput quantitative nanoscopy imaging, with the ability to achieve statistical significance in SMLM for both PAINT and PALM techniques.

INTRODUCTION

Single molecule localization microscopy (SMLM) enables detailed visualization and study of microbial structures, subcellular components, molecular interactions, and cellular processes at nanoscale resolution. Considering the modest size and complex internal organization of small organisms like bacteria, SMLM represents a key investigative technique. However, achieving sufficient throughput with traditional SMLM techniques is time-consuming and challenging. Bacterial populations are heterogeneous, and imaging only a few cells limits understanding of diverse cellular behaviors. Additionally, small samples lack statistical significance and can introduce biases.

Abbelight proposes adaptive scanning for tunable excitation regions (**ASTER**)[1] to achieve a uniform and large field of illumination for quantitative and high-throughput nanoscopy imaging. This method enables high-throughput imaging of bacterial cell components at a fine scale with SMLM techniques such as photoactivation localization microscopy (PALM)[2] and point accumulation for imaging in nanoscale topography (PAINT)[3].

Abbelight Platform Advantages:

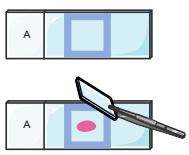
- **Time-saving:** Imaging hundreds of cells in a single field rapidly generates more data per acquisition.
- **Statistical significance:** Uniform large FOV illumination avoids heterogeneity in the sample.
- **Broader sampling:** Provides a more representative view of the entire population.
- **Quantitative analysis:** Allows illumination and detection of each molecule across the entire region for statistical analysis.
- **Adaptable:** Access to different illumination angles makes it usable for dSTORM, PAINT, PALM, and TIRFM techniques.
- **Resource efficient:** Saves time, manpower, equipment, and finances for the research project..

WORKFLOW

The Abbelight imaging platform aims to offer a user-friendly experience from sample preparation to image analysis with confidence, allowing better sampling of the entire population in a shorter time (**Figure 1**).

Abbelight Imaging Platform: PALM

1. Sample preparation



Immobilization of bacteria on a gel with **Abbelight™ Pad Kit**

2. PALM Acquisition

Full control of acquisition via **Abbelight™ NEO** Imaging software

3. Imaging

Multi model imaging for SMLM and TIRFM with **Abbelight™ SAFe MN 360**

4. Analysis

Post processing, filtering, DBSCAN clustering and rendering using **Abbelight™ NEO** Analysis

Figure 1: Abbelight offers streamlined workflows, from sample preparation to image analysis.

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RESULTS

Achieving Uniform Illumination with ASTER

High-throughput nanoscopy imaging can be very challenging. It requires imaging multiple small ROIs of ~50 x 50 µm² (the market standard) to achieve a couple of hundred imaged cells. It is also time-consuming for such a small region, which can vary from 5 to 30 minutes for imaging at PALM and PAINT, respectively. Abbelight ASTER provides the largest field of view (FOV) with uniform illumination up to 150 x 150 µm² with a 100x objective in SMLM. This makes it possible to image a couple of hundred cells per acquisition, saving time and providing a complete picture of a population in a single image.

We used the Gram-negative model bacterium *Escherichia coli* to visualize the bacterial membrane labeled with Potomac Gold using PAINT (**Figure 2**). Potomac Gold binds transiently to the bacterial membrane and allows us to image the *E. coli* cell envelope at a fine scale [3]. As a result, we imaged 843 cells and the complete structure of each one of them in a single large field of 150 x 150 µm², while it could be only 71 cells in the standard market FOV of 50 x 50 µm² (**Figure 2**, green square). This technique can be combined with the imaging of different proteins or structures to resolve subcellular mechanisms of such small organisms in high resolution.

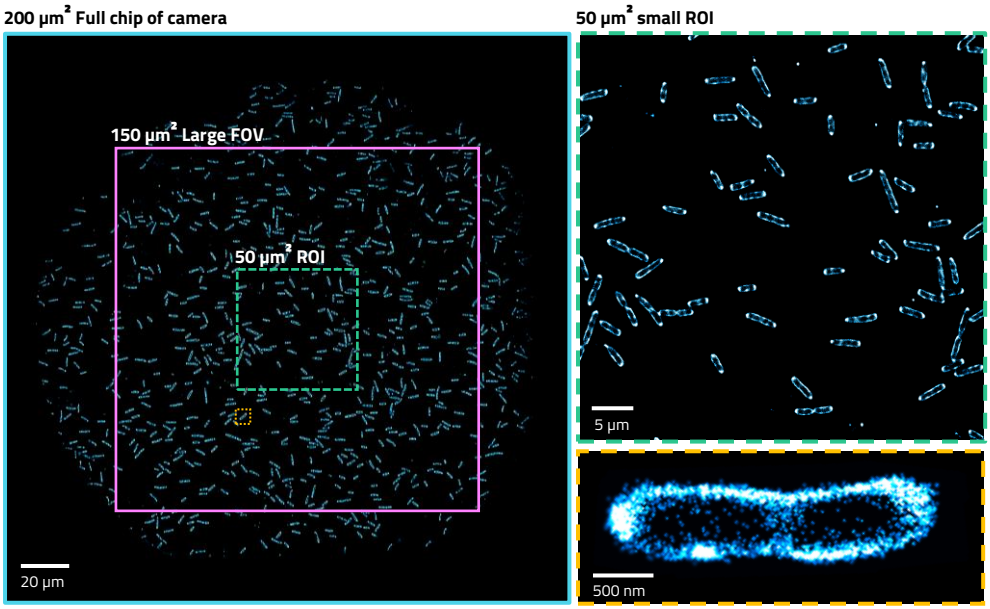

Figure 2: Large FOV PAINT imaging of *Escherichia coli* cells for quantitative and high throughput analysis. *E. coli* cells were labeled with the spontaneously blinking dye Potomac Gold, which targets the bacterial membrane. The full chip of the camera with an area of 200 µm², the large FOV of 150 µm², and the ROI of 50 µm² are shown in turquoise, pink, and green squares, respectively. The orange dotted square indicates a dividing single *E. coli* cell. A total of 10 000 frames were acquired, and 10 111 537 molecules were detected. The total acquisition time was 4 minutes and 25 seconds.

Uniform Illumination at a Large FOV

Uniform illumination on the sample is crucial for unbiased detection of the protein composition per cell/unit to generate reliable and robust statistics. This is especially important for quantitative image analysis, where the number of detected molecules per unit carries statistical significance. Quantitative analysis can only be achieved with PALM by using photoactivatable fluorescent proteins such as PAmCherry1 and PA-GFP[2,4,5,6]. Combining PALM with Abbelight's ASTER ensures uniformity over the samples at nanoscale resolution, resulting in strong statistical significance for high-throughput quantitative image analysis (**Figure 3**).

To observe the uniform illumination and detection over a large field, we imaged PAmCherry1 expression at the curvature of Gram-negative live bacteria *Rhodospirillum*. A total of 148 cells were imaged in a single large region of 150 x 150 µm². Curvature localization can be observed in every single cell from every corner of the field, thanks to uniform illumination.

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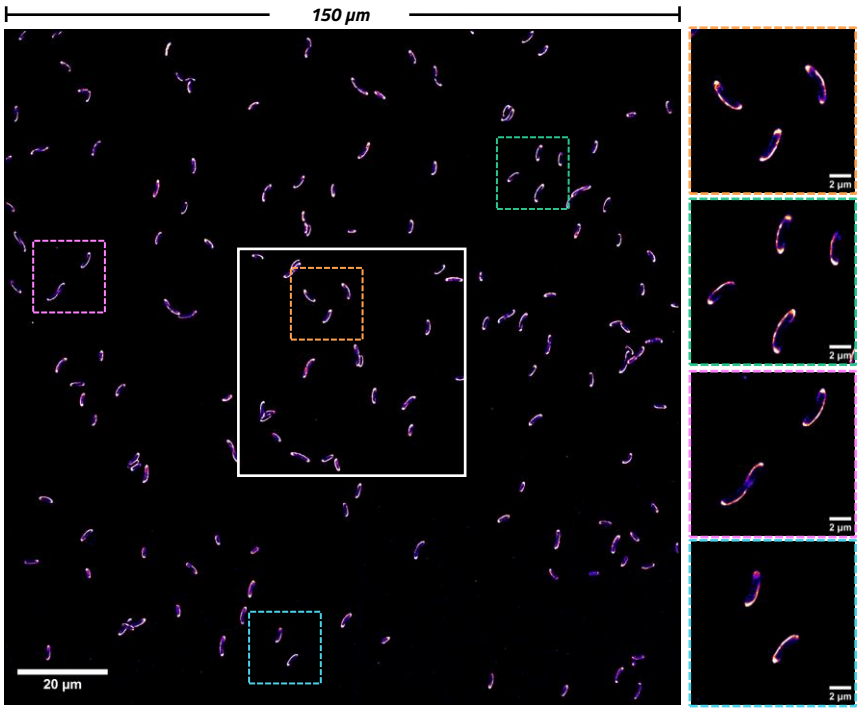

Figure 3: Uniform large FOV PALM used to visualize the curvature protein of *Rhodospirillum.* The PALM technique is carried out by pulsed activation of 405 nm and continuous excitation of 561 nm lasers to image PAmCherry1 molecules. The *Rhodospirillum* curvature protein is labeled with PAmCherry1. Uniform HiLO illumination is used for a large field of view (FOV) of 150 x 150 µm² in the ROI. Orange, green, pink, and turquoise squares show small ROIs from different regions of the large FOV. The white square represents a 50 x 50 µm² region for market standards.

Quantitative Image Analysis

PAmCherry1 is activated by exposure to UV light, permanently switching from a non-fluorescent state to a red fluorescent state and cannot revert to its original non-fluorescent form. This irreversible cycle of activation and imaging of each molecule allows us to quantify the total number of molecules per cell. To further understand the localization of the curvature protein, we quantified the number of molecules per cell area. Due to the bacterium's increasing size until division, we adjusted our results from younger to older cells. Older cells, which are larger and closer to division, were compared with younger, smaller cells.

Cluster analysis with the DBSCAN algorithm was used to segment each cell. A cluster table, including cell identity number, centroid position, number of localizations, volume, and density, was exported from Neo Analysis software. Twenty-four cells were discarded from the analysis due to incomplete cell shapes at the edges. The remaining 124 cells were investigated based on cell size and the number of molecules per cell. The number of molecules per cell was found to increase with the size of *Rhodospirillum* cells. Mature cells closer to cell division contained an average of 6.4K molecules, whereas younger cells contained an average of 4K molecules (see **Figure 4**). The spiral shape of *Rhodospirillum* was easily observed within the mature cells (see **Figure 4**, middle). In young cells, 3 343 molecules were detected within a 1.015 µm² area, while nearly three times more molecules were detected in older cells within a 4.88 µm² area (Figure 4).

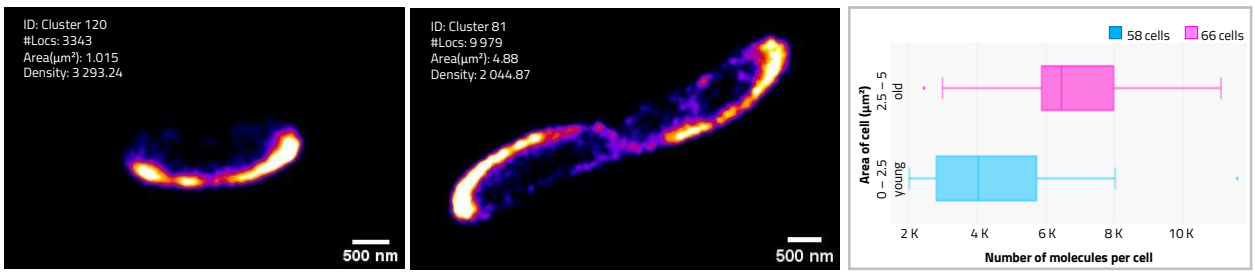

Figure 4: Resolving spiral-shape of *Rhodospirillum* and quantification of curvature targeting protein. Curvature protein labeled with PAmCherry1 for quantitative PALM imaging. Single cells with smaller (Left) and larger areas (Middle) were chosen to represent young and old cells. Cluster information is included in the top left corner of each cell. Box plot comparison of molecule localization in young (cyan) and old (magenta) cells. Error bars show 10th and 90th percentiles.

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CONCLUSIONS

Here, we demonstrate that uniform illumination enables achieving statistical significance in SMLM for both PAINT and PALM techniques. This represents a significant advantage for studying diverse bacterial populations, facilitating the imaging of hundreds of bacterial cells in a single acquisition with fine-scale localization of molecules in each cell. A large field of uniform illumination enables better sampling of the entire population, saving time and resources, as well as improving the statistical power of quantitative imaging.

The Abbelight imaging platform aims to offer a user-friendly experience, from sample preparation to image analysis with confidence. Contact us to learn more about our products and offerings.

METHODS

SAMPLE PREPARATION

Fixed *E. coli* cells were provided by Dr. Christoph K. Spahn. Live *Rhodospirillum* cells were prepared by Dr. Rogelio Hernandez at Prof. Dr. Martin Thanbichler lab.
PAINT: Bacteria were immobilized on an Ibidi glass bottom 8-well dish with a 1.5H thickness. Immobilized cells were permeabilized with Triton X-100 in PBS. 600 pM Potomac Gold in PBS was added to each well and PAINT imaging was performed as described in Spahn et al.'s 2018 Scientific Reports.
PALM: The immobilization gel was prepared according to the protocol of the **Abbelight™ Pad Kit** for live cell imaging.

IMAGING

PALM imaging was performed on the **Abbelight™ SAFe MN 180** instrument combined with a Nikon Eclipse Ti2-E microscope body. A 100x Apo TIRF oil objective and a perfect focus system were used for imaging.
PAINT imaging was performed on the **Abbelight™ SAFe MN 360** instrument combined with an Olympus IX83 microscope body. A 100x Apo TIRF oil objective and a ZDC continuous focus system were used for imaging.
All live imaging and reconstruction parameters were set using **Abbelight™ NEO** Live imaging software v2.16.3. Output files included raw acquisition ROI.tif, drift table .csv, and nanoscale reconstructed image .tif for post-processing.

ANALYSIS

Abbelight™ NEO Analysis v38 was used for post-processing and reconstruction of molecule localizations. The Gaussian Fitting Maximum Likelihood Estimation (MLE) algorithm was used for localization. Cross-correlation was employed for drift correction during post-processing. The DBSCAN algorithm was used for clustering and quantitative analysis within the NEO Analysis software.

ACKNOWLEDGEMENTS

We thank Prof. Dr. Martin Thanbichler and Thanbichler Lab members for providing *Rhodospirillum* cells and performing imaging with their **Abbelight™ SAFe MN 180** system at Marburg MPI. We thank also Dr. Christoph K. Spahn for providing fixed *E. coli* cells and sharing PAINT protocol for staining and imaging bacterial membranes.

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Abbelight

Founded in 2016, Abbelight is a fast-growing company specialized in imaging solutions focusing on microscopy and unique single molecule detection (super-resolution).

The portfolio integrates a constantly evolving know-how on chemistry, optics and computer science to offer a complete solution, from sample preparation to data management, including an optimal bio-imaging platform that can be adapted to all researchers', biotech labs' and medical facilities' needs.

Abbelight is a French company developed by four passionate researchers who aim to help improve human health in various areas such as bacteriology, extracellular vesicles, neurosciences, structural biology...

Today, Abbelight employs over 60 people who are all driven by the goal of providing the best solutions and support to our customers all around the world.

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