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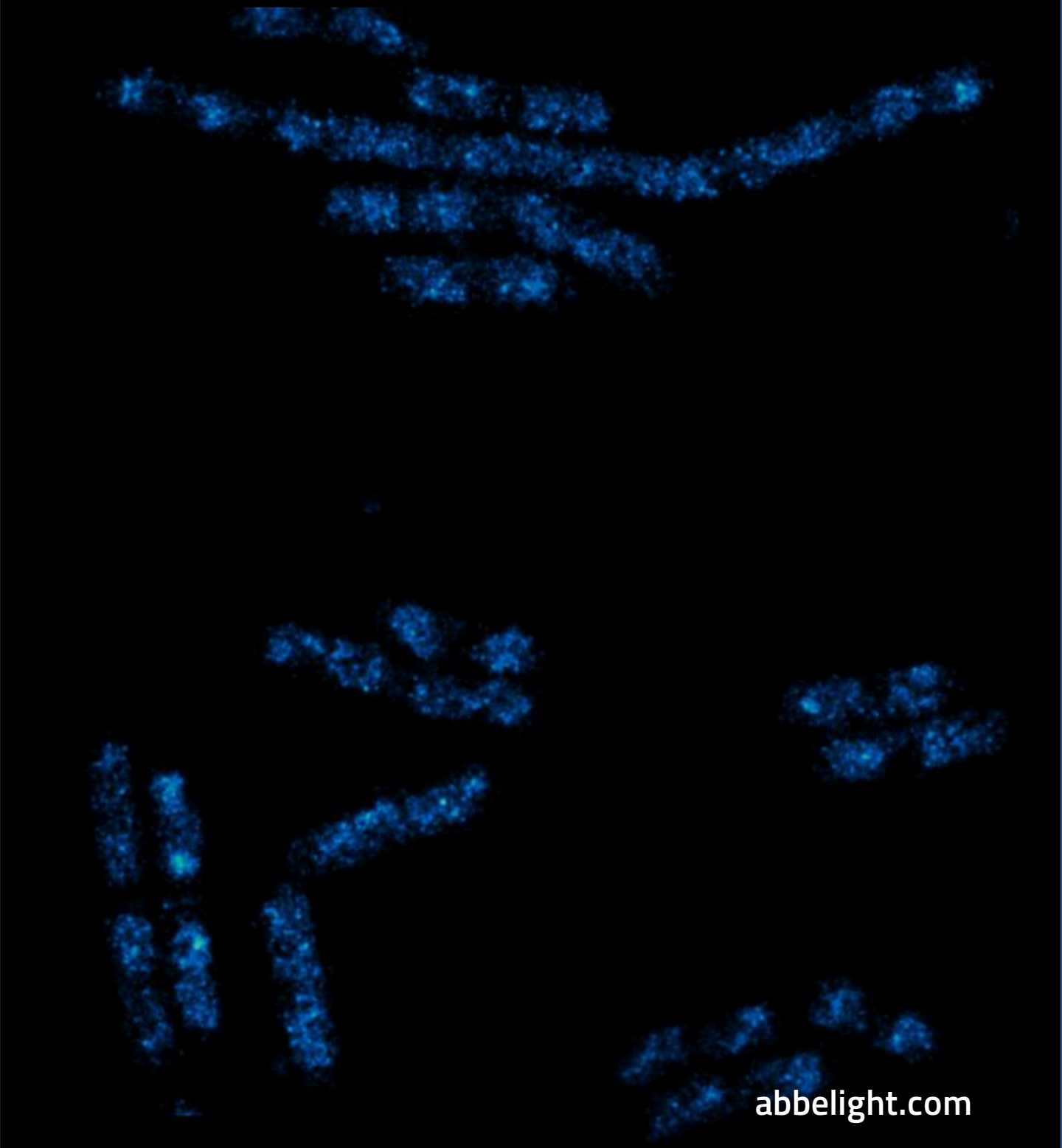
Nanoscale spatial organization and dynamics of E. coli RNA Polymerase by PALM microscopy



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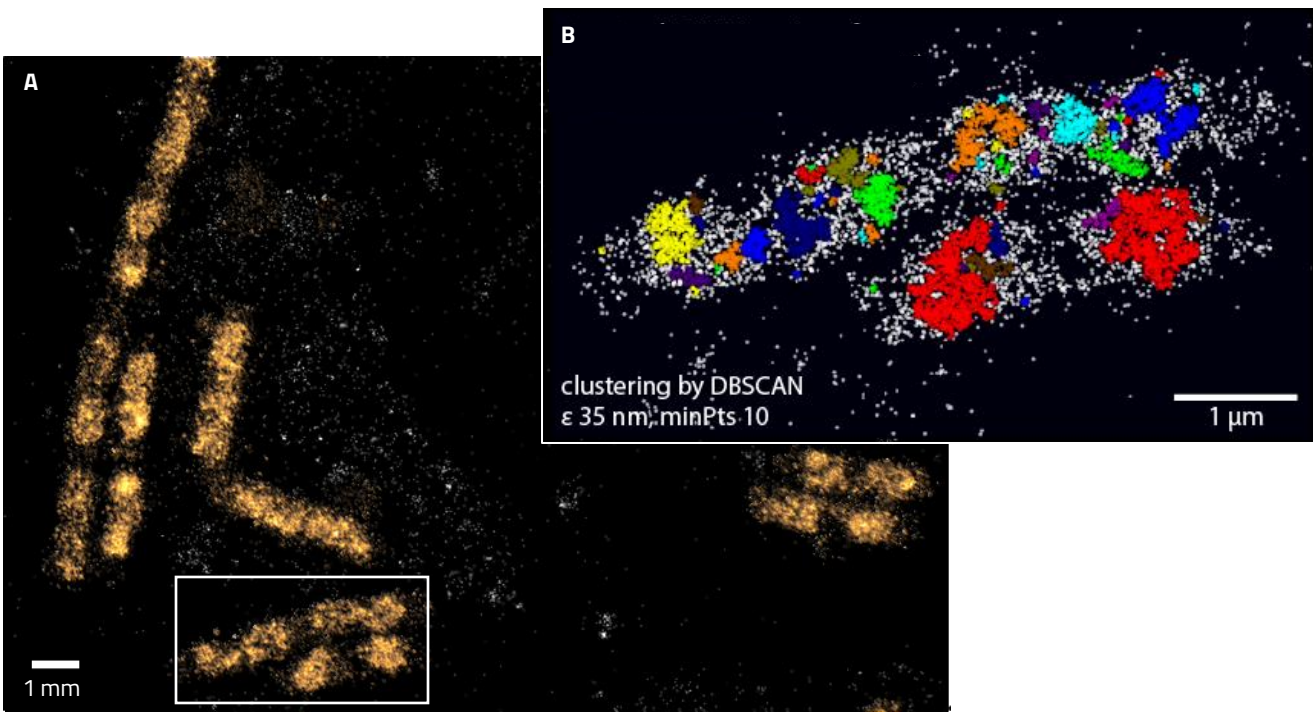
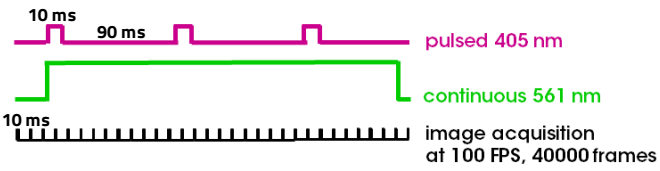
Nanoscale spatial organization and dynamics of *E. coli* RNA Polymerase by PALM microscopy

Photoactivated Localization Microscopy, or PALM imaging [1] exploits the unique behavior of fluorescent proteins (FPs), whose spectral properties change upon exposure to specific wavelengths of light. Upon illumination with the activation light a random and sparse number of FPs are activated/photoconverted and emit fluorescence when exposed to the excitation light. The light emission from individual fluorophores can be recorded and used to localize and track single molecules with sub-diffraction precision, as in other SMLM super-resolution techniques. By concatenation of activation/detection/bleaching steps all the FPs in the sample can be counted, localized and tracked over time. PALM FPs can be appended to other proteins via genetic engineering and thus allow super resolution imaging of virtually any protein-of-interest within the cell, independently of the availability of antibodies or other affinity reagents.
In fast-growing *Escherichia coli* cells, RNA polymerase (RNAP) is spatially organized in large nucleoid-like patterns, which have been proposed to be active transcription centers for ribosomal RNA (rRNA) [2, 3]. Here, we show how PALM super resolution imaging and single particle tracking (sptPALM) can be used to dissect the nanoscale organization and dynamics of *E.coli* RNAP and gain insights into the mechanisms of bacterial transcription regulation.

Structural PALM imaging of *E. coli* RNAP in chemically fixed cells



E. coli cells expressing Dendra2-tagged endogenous RNAP (rpoC-Dendra2) [4] were cultured to their early exponential growth phase (OD 0.2) and chemically fixed with aldehydes. PALM super resolution and density-based clustering (DBSCAN) [2, 5] were used to characterize the structural organization of RNAP in *E. coli* cells.
Dendra2 molecules were photoconverted and detected at 10 ms per frame using the following illumination scheme: the 561 nm readout laser was set to maximum power and continuous emission (about 38.5 mW at the sample plane); the 405 nm photoconversion laser was pulsed every 10th frame, and its power gradually increased from about 2 mW throughout imaging to keep the number of fluorescent events at a constant level.



In A, a reconstructed image from a PALM super resolved dataset of localized Dendra2-RNAP (median precision of localization of 15 nm); the localization events within the bacterial cells are highlighted in orange.
In B, two representative fixed *E. coli* cells with Dendra2-tagged RNAP molecules grouped into clusters using DBSCAN [5], applied using an epsilon of 35 nm and a MinPts of 10 (optimal values taken from [2]).

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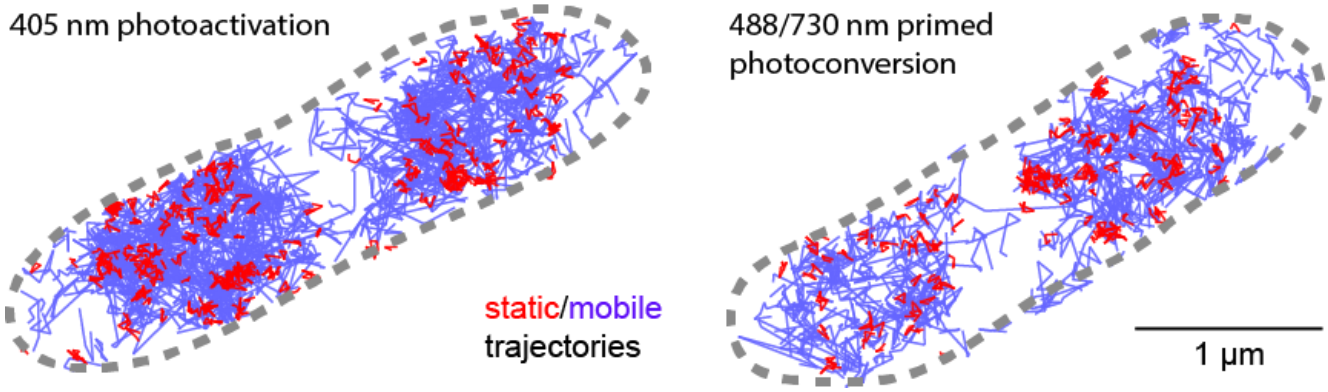
Single-particle tracking (spt)PALM imaging of RNAP in living *E. coli* cells.

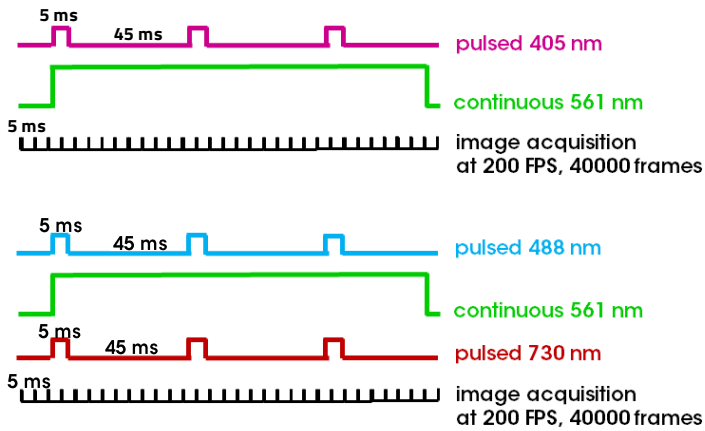


E. coli expressing Dendra2-RNAP were cultured as described and imaged live, at a frame rate of 200 FPS (5 ms per frame). Single-particle tracking (spt)PALM was used to characterize the dynamic behavior of individual RNAP molecules. Two representative *E. coli* cells are shown with RNAP trajectories colored by their motion type being either static (transcribing, red) or mobile (diffusive, blue).

For sptPALM imaging, the 561 nm readout laser was set to its maximum power and continuous emission (about 38.5 mW at the sample plane). To photoconvert Dendra2 molecules into their red fluorescent state, two alternative photoconversion modes were used [4, 6]: **UV-photoconversion** by 405 nm light or by dual illumination of blue (488 nm) and near-infrared (730 nm) light, a photoconversion mode known as **primed photoconversion** [6], newly discovered in green-to-red photoconvertible FPs from the family of Anthozoans that possess a threonine at position 69, such as Dendra2 [4].

1. UV-photoconversion: A 405 nm laser was pulsed to emit every 10th imaging frame. Its laser power was gradually increased from initially about 2 mW throughout imaging to keep the number of fluorescent events at a constant level.

2. Primed photoconversion: A 488 nm and a 730 nm laser were pulsed to emit every 10th imaging frame. The 488 nm power was gradually increased from initially about 6 mW throughout imaging to keep the number of fluorescent events at a constant level, while the 730 nm laser intensity was set to its maximum power of about 4.5 mW. It must be noted that a higher intensity of the 730 nm laser is needed to achieve primed photoconversion of Dendra2 in fixed cells (at least 10 mW at the sample plane, not available for the experiments presented here).



Both modes yield efficient and quantitative read-out of the FP labels [4]. In a direct comparison, primed photoconversion has some advantages: It allows for less phototoxic sptPALM imaging [4] and can be combined with photoactivatable proteins (e.g., PAmCherry) to allow for dual-color sptPALM and PALM imaging. This new dual-color read-out scheme offers – due to its genetic nature - highly specific labeling and allows for a quantitative and aberration-free read-out of FP signals [7].

Data and text courtesy of **Bartosz Turkowyd** & **Ulrike Endesfelder**

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Methods

PREPARATION

The C-terminus of the subunit rpoC of RNAP was tagged by the fluorescent protein Dendra2 at its endogenous genetic locus, as described in [4]. Cells were cultured in a rich medium and harvested in their early exponential growth phase (OD 0.2).

Cells were imaged either live, for **sptPALM** tracking experiments, or fixed (4% paraformaldehyde for 15 minutes at room temperature) for **structural PALM** imaging.

IMAGING

Single-molecule imaging was performed on an Olympus Ix83 microscope with a 100X 1.49NA objective, equipped with **Abbelight™ SAFe360** that implements **ASTER** technology for homogeneous TIRF and HILO illumination over a large field of view.

Single-molecule fluorescence was detected on ORCA-Flash 4.0 v3 sCMOS cameras (Hamamatsu) running at 10 ms (fixed samples) and 5 ms (live samples) per frame, and with a pixel size of 97 nm. Laser illumination and image acquisition were controlled with **Abbelight ™ Neo** imaging software.

ANALYSIS

Single molecule localization, drift correction, post-processing, image reconstruction and 3D visualization were done using **Abbelight ™ NEO** software.

Abbelight ™ NEO was used for further analysis of **structural PALM** datasets from fixed cells: localized events were filtered for uncertainty of the localization and events coming from the same emitter were merged; the resulting datasets were then analyzed using **DBSCAN** [5] for clustering of RNAP at the nanoscale.

For **sptPALM** analysis, the Abbelight software was used for the initial postprocessing steps, with the exclusion of drift correction; unmerged localization events were exported, tracking and motion type inference was performed using the *swift* tracking software [8].

Highlights

What are the advantages of PALM super resolution imaging?

- *Versatility and **specificity** through genetic tagging*
- *Allows **live cell super–resolution imaging***
- ***Quantitative** clustering of proteins at the nanoscale.*
- ***Dynamic** tracking of single molecules inside cells (sptPALM).*
- *Highly specific quantitative and aberration-free dual-color live cell imaging via **primed photoconversion***

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swift software available for beta-testers upon request!

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