Correlative super-resolution imaging of RNA polymerase distribution and dynamics, bacterial membrane and chromosomal structure in *Escherichia coli*

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1. Introduction

Single-molecule localization microscopy (SMLM) allows visualization of biological processes occurring at scales far below the diffraction limit of light. SMLM temporally confines the fluorescence signal by making use of photoactivatable or photoswitchable fluorescent probes [1–3]. Since the introduction of this imaging concept, various developments have paved the way for SMLM today being a common and reliable technique used to solve biological questions [4]. This includes the development of novel probes with superior photophysical properties [5–8], advanced imaging schemes [9–14], faster or more sensitive cameras [15–17] and algorithms ([18] and International Symposium on Biomedical Imaging challenge (http://bigwww.epfl.ch/smlm/)).

SMLM is very well suited to visualize and understand cellular processes in prokaryotes, which are otherwise difficult to access because of their small size. SMLM can provide maps of protein organization and cellular structures at the near molecular-level, with a spatial resolution approaching 20 nm and with single-molecule sensitivity. In addition, localization-based algorithms provide access to sub-populations and heterogeneities that are otherwise hidden by ensemble averaging. Using these approaches, various groups have reported information on protein copy numbers and distributions in bacterial cells [19–22], and have studied the dynamics of cellular processes in living cells [23–27].

For targeting proteins and other biomolecules in densely packed bacterial cells, different labeling approaches have been established. Live cell imaging and quantification of endogenous proteins are commonly carried out using fluorescent proteins [19, 24–29]. The bacterial cell surface can be visualized by using phase contrast brightfield microscopy [30] or reversible binding of fluorophores to the lipid bilayer [31, 32].
Fluorophores suitable for super-resolution microscopy can also be covalently linked to the medium-exposed proteins on the cell surface with the use of NHS-functionalized dyes [33] or by introduction and labeling of non-natural amino acids designed for 'click chemistry' [34, 35]. Click chemistry also facilitates direct targeting of DNA into which the thymidine analogue—5-ethyl-2′-deoxyuridine (EdU) was incorporated during replication [22, 36]. Although these individual approaches are well established, multi-targeting imaging schemes for prokaryotic cells have to date remained demanding, as an applicable set of fluorophores and orthogonal labeling strategies still are challenging.

We here introduce a multi-color imaging strategy combing sequential PALM (photoactivated localization microscopy) [3] or single particle tracking of a protein of interest, PAINT (point accumulation for imaging in nanoscale topography) [31, 32] visualization of the intact membrane and subsequent click chemistry labeling and dSTORM (direct stochastic optical reconstruction microscopy) [37] acquisition of the DNA based on two different commercial available fluorophore combinations.

The driving force behind the development of sequential super-resolution imaging was the desire to visualize bacterial proteins that interact with the bacterial chromosome, the structure of the bacterial nucleoid and their positioning within an individual cell using single-molecule super-resolution microscopy [1]. Here, quantitative PALM imaging of the fusion protein of the endogenous RNA polymerase and PamCherry1 was performed, while the chromosomal DNA is imaged in high resolution by applying a previously published protocol for high-density labeling using the 'click chemistry' reaction [22, 36, 38]. However, and up to now, the most efficient click reaction requires catalysis by copper (1), which drastically reduces the pool of fluorescent proteins that still exhibit fluorescence emission. The chemicals in the imaging buffer used for dSTORM imaging additionally impair the photophysical properties of photomodulatable proteins, as they do not tolerate high concentrations of thiols or oxygen depletion [39]. The 'snapshots' generated by our sequential approach can be assigned to the cell cycle stage according to the length of the bacterium, for which it is crucial to obtain a precise microscopic image of the bacterial cell membrane. However, bright-field (BF) images are blurred because of the diffraction of light while membrane permeabilization, which is required for click chemistry labeling of the nucleoid, impairs phase-contrast microscopy and PAINT imaging. To solve this problem, we perform PAINT imaging after PALM imaging and before membrane permeabilization in a sequential workflow which allows us to extract the exact cell boundaries.

Using this sequential approach, we found that RNA polymerase populates preferentially the edge regions of the highly structured nucleoids during fast growth, and is spread more uniformly within the cytosol during slower growth, both in agreement with former studies [20, 40], Furthermore, we observed highly condensed regions which are inaccessible to RNAP molecules both in fixed and in living E. coli cells.

2. Results

2.1. Sequential super-resolution imaging

We developed an experimental protocol that complies with the above requirements and constraints of multi-color super-resolution imaging of E. coli (figure 1). Bacterial cells were grown in media containing the artificial nucleobase 5-ethyl-2′-deoxyuridine (EdU). After membrane-conserving fixation, cells were immobilized on a glass substrate and covered with a hydrophilic gel matrix [41]. PALM images of RNA polymerase (RNAP), tagged with the photoactivatable fluorescent protein PamCherry1 (strain KF26) [20], were recorded at multiple regions on the glass slide. Their relative positions to a prominent spot on the chamber were saved for re-localization of the imaged bacteria (figure S1 (stacks.iop.org/MAF/3/014005)). We then added low concentration (typically 100–500 pM) of the PAINT-dyes Nile Red or Rhodamine 6G (R6G), which allow ‘painting’ the boundaries of the bacterial cell [31] by reversibly binding to the intact membrane. As PAINT imaging can be performed using different suitable dyes, the approach can be adapted to available laser lines. Nile Red images can be obtained by
illumination with 561 or 568 nm excitation light and are shown in figure 1. R6G images using a 532 nm excitation light can be found in the supplemental information (figure S2 (stacks.iop.org/MAF/3/014005)). After acquiring all bacterial outlines we performed the click chemistry reaction on-the-slide as described in the methods section. This facilitated recording dSTORM images and visualizing the bacterial chromosome with sub-diffraction resolution.

Imaging of a larger field of view with multiple bacterial cells (4–8) allows for a simple and straightforward overlay of the individual images based on the shape and orientation of all cells (figure 2). Drift correction and image registration are discussed in the supplemental information (figures S3 and S4 (stacks.iop.org/MAF/3/014005) and supplemental methods). PALM (figure 2(b)), PAINT (figure 2(c)) and dSTORM images (figure 2(d)) can be combined with each other and overlaid with the respective brightfield image (figure 2(a)). Overlaying brightfield and PAINT images (figure 2(e)) can be useful as a calibration if bacterial geometry should be determined via brightfield-images. Combining the PAINT- and the dSTORM images (figure 2(f)) provides insights into the spatial distribution and positioning of the nucleoids within the cell, whereas the overlay of PALM and dSTORM images shows the spatial arrangement of RNAP and the nucleoids (figure 2(g)). The distribution of RNAP and DNA within the cell can be investigated by combining all imaging channels (figure 2(h)). From these images, we calculated the experimental localization precision using a nearest-neighbor approach [42] and obtained values of 12.0 nm (PALM), 22.1 nm (PAINT) and 12.6 nm (dSTORM) (figure S5 (stacks.iop.org/MAF/3/014005)).

Using this sequential imaging approach, we investigated the spatial distribution and correlation of RNAP and DNA during the cell cycle of E. coli grown under two different conditions (figures 3 and S4 (stacks.iop.org/MAF/3/014005)), using LB as nutrient-rich (doubling time 25 min; figure S6 (stacks.iop.org/MAF/3/014005)) and M9 + 0.2% glucose + 0.1% casamino acids as nutrient-poor (doubling time 44 min; figure S6 (stacks.iop.org/MAF/3/014005)) medium. We observe the typical patterns for RNAP as reported in earlier work [20] (figure 3(a)): RNAP segregates in up to four distinct bands during fast growth (LB), containing ‘hot spots’ with high protein content. In bacteria grown at slower growth rates (M9), RNAP is distributed more uniformly (figure S7(a) (stacks.iop.org/MAF/3/014005)) within the cell volume (figure S7(b) (stacks.iop.org/MAF/3/014005)).

The bacterial nucleoids are found to be strikingly structured in LB medium, exhibiting thin filaments and DNA condensation centers (figure 3(c)) as reported before [22]. Similar to RNAP, up to four well-separated ‘bands’, which represent the single chromosomes, are found at quarter positions on the bacterial length axis. During the cell cycle, the nucleoids increase in length until they split, which constitutes the concomitant nature of replication and DNA-segregation (figures 3(c) and S8 (stacks.iop.org/MAF/3/014005)). RNAP and DNA share those regions, since RNAP is to a large fraction bound to...
DNA in order to mediate transcription. However, we observe that RNAP is mostly located at the periphery of the dense chromatin and in small gaps between the condensed chromosome regions. We assume that active genes are exposed out of the condensed chromatin, where they are accessible for RNAP and transcription. This state is maintained during the whole cell cycle, assuring that an equal amount of the RNAP pool is passed to the daughter cells upon cell division (figures 3 and S8 (stacks.iop.org/MAF/3/014005)) [40].

At slower growth rates (M9 medium), bacteria show a much less pronounced nucleoid structure (figure S4(c) (stacks.iop.org/MAF/3/014005)) with no global structural changes during the cell cycle that would be accessible to our imaging methods. The nucleoid splits into two daughter chromosomes, while bulk DNA is concentrated in the middle of the cell compared to the membrane-association in LB (figures 4 and S7(c) (stacks.iop.org/MAF/3/014005)). For RNAP, less obvious bands with a lower molecule density at sites of future cell division are observed. RNAP is also less concentrated at the nucleoid edges but populates the whole cytoplasmic space. However, the nucleoids seem to possess highly condensed cores which are inaccessible to RNAP (figure 4). Studies investigating even slower growth rates showed that the chromosomes are even less condensed and therefore populate a bigger volume relative to the whole cell volume [43, 44].

RNAP is thought to affect or even induce compaction and structuring of the nucleoid [40]. During fast growth most of the RNAP molecules transcribe rrn operons. The RNAP pool available for transcription of other genes is thus depleted, which might in turn lead to high chromatin compaction mediated by domain-ins [45]. At slower growth rates, less rrn genes are transcribed, leading to increased transcription of non-ribosomal genes along the whole chromosome and a more uniform distribution of RNAP within the cell.

2.2. Sequential single-particle tracking and super-resolution imaging

We developed a second protocol that allows extracting dynamic information of bacterial proteins from single-molecule trajectories, and combining these with ultra-structural information of the chromosome. A combination of sptPALM and dSTORM was reported in mammalian cells [14]. Relating the bacterial RNAP dynamics to the structural DNA organization is challenging, as high densities and imaging tools with both a high spatial and temporal resolution are needed. The correlative and sequential protocol first tracks the dynamic RNAP molecules via sptPALM, followed by the structural study of the bacterial chromosome via dSTORM. This allows to link the different diffusional states of RNAP (e.g. actively transcribing versus stagnant) to the underlying positioning on the DNA. KF26 was grown and imaged in EdU containing medium to allow labeling of the nucleoid with a suitable fluorophore for dSTORM imaging at a later stage. The living bacteria were immobilized on a poly-L-lysine coated glass slide and single-molecule trajectories were recorded according to the single-particle tracking PALM (sptPALM) principle [46] (figure 5(a)). After read-out of all RNAP trajectories, the cells were immediately fixed to prevent chromosome remodeling, permeabilized and DNA was click-labeled on-the-slide. The highly resolved dSTORM-images of the nucleoids (figure 5(b)) were registered with the RNAP trajectories (figure 5(c)). Due to the immobilization procedure at room temperature bacteria exhibited a medium fast growth rate. Here, the correlation of RNAP dynamics to the nucleoid structure matches our observations in fixed cells grown in M9 (figure S7 (stacks.iop.org/MAF/3/014005)). The nucleoid appears relatively unstructured and does not cover the complete bacterial cross axis while RNAP molecules mostly move along tracks at the edge of or outside the dense chromatin regions. Dense nucleoid centers are also inaccessible for RNAP as seen in fixed cells (figure 5).
3. Conclusions

We demonstrate two easily applicable and useful experimental protocols for correlative imaging of protein distribution and dynamics, membrane and chromosome structure in bacterial cells. We applied the protocols to study the spatial pattern of RNAP and chromosomal DNA in two different growth media. We were able to reproduce results of two separate previous studies, but for the same individual cells by combining PALM and dSTORM. Correlating the dynamics of RNAP in live bacterial cells with highly resolved nucleoids gives insights into the protein dynamics. It will be strikingly interesting to apply these protocols to other nucleoid-associated proteins, different media and drug treated cells in order to better understand the interactions and complex mechanism occurring at such a small scale.

4. Methods section

4.1. Cell culture

KF26 [20] was inoculated from glycerol stocks and grown over night (ON) in the respective media at 32 °C and 200 rpm shaking. Working cultures were inoculated 1 : 200 from ON cultures and OD_{\text{600}} was determined every 30 min to obtain the doubling time for each culture (figure S2 (stacks.iop.org/MAF/3/014005)), which was determined to be 25 min in LB medium and 44 min in M9 medium containing 0.2% glucose, 1 μg ml^{-1} thiamine and 0.1% casamino acids (all from Sigma). EdU (Baseclick) was added at 10 μM concentration for 40 min to the LB culture at an OD_{\text{600}} of ~0.25 to cover the time needed for one complete replication round. Due to overlapping replication cycles, EdU-incorporation thus covers the whole chromosome. In order to achieve similar labeling in M9 medium, EdU was added for 66 min (1.5 mass doubling-times) at a similar OD_{\text{600}}. 100 μg ml^{-1} Ampicillin was added to each culture for selection.

4.2. Fixation and immobilization

To maintain positions of immobilized bacteria and prohibit detachment from the glass slide, we added a hydrophilic gel matrix to the sample [41]. Extracellular matrix (ECM) gel from Engelbreth-Holm- Swarm murine sarcoma (Sigma) at a protein concentration of 15 mM and 1%, respectively. After 30 min, the cells were pelleted for 3 min at 5,000 × g and washed once with 100 mM sodium-phosphate-buffer pH 7.5 containing 50 mM ammonium-chloride to reduce excess formaldehyde. Resuspended bacteria were then immobilized on KOH-cleaned (3 M, 30 min), poly-L-lysine-coated 8-well-chamberslides (Sarstedt) and washed twice with phosphate buffer. Buffer was then removed and the sample was placed 5 min on ice before proceeding with gel matrix coating.

4.3. Coating and post-labeling

To maintain positions of immobilized bacteria and prohibit detachment from the glass slide, we added a hydrophilic gel matrix to the sample [41]. Extracellular matrix (ECM) gel from Engelbreth-Holm- Swarm murine sarcoma (Sigma) at a protein concentration...
of 3.5–4.5 mg ml\(^{-1}\) (1 : 1 dilution in ddH\(_2\)O) forms a porous matrix which facilitates post-labeling procedures. The ECM gel was thawed on ice and then diluted with ice-cold ddH\(_2\)O using pre-chilled pipet tips. 30 \(\mu\)l of diluted gel was added to the immobilized bacteria. Subsequently, the whole sample was first incubated 5 min on ice for proper sample coverage and then incubated at 37 °C for 30–40 min for gel polymerization. Final post-fixation with 1% FA in 100 mM sodium-phosphate buffer for 15 min prevents the re-liquidation of the gel at low temperatures. The sample was washed once with phosphate-buffer containing 50 mM ammonium-chloride and transferred to the microscope for PALM-imaging (see below). After PALM-imaging of all desired regions, Nile Red was added at 100–500 pM concentration for PAINT-imaging of the bacterial membrane of the same regions. After 2 washing steps, the membrane was permeabilized with 0.5% TritonX-100 in phosphate-buffer for 60 min and DNA was labeled via click chemistry for further 60 min as reported elsewhere [47]. The sample was washed carefully 3 times with phosphate buffer for 5 min and 2 more times for 2 h to remove excess dye-azide.

### 4.4. Super-resolution imaging

All samples were imaged on a custom build setup for single-molecule detection. In brief, a Coherent Innova 70C Spectrum and a Coherent Cube UV-laser (404 nm/100 mW) laser are coupled into a 100 × Apo TIRF oil objective (NA 1.49), mounted on a Nikon Eclipse Ti microscope body. The z-position is maintained using a perfect focus system (Ti-PFS; Nikon), the x,y-position is adjusted with a mechanical stage (Nikon). Fluorescence light passes the dichroic mirror (F73-888; AHF) and is finally detected on an Andor Ixon Ultra EMCCD chip (DU-897U-CS0-#BV; Andor) using a preamplifier gain of 3 and EM-gain of 200. Excitation laser lines are selected using an acousto optic tunable filter (AOTF; AA Opto Electronic) and illumination mode can be changed from widefield- to TIRF-illumination using a manually adjustable TIRF mirror. Camera and microscope were controlled with the \(\mu\)Manager software [48].

PALM-imaging of RNAP-PamCherry1 in fixed cells was performed using 568 nm excitation (1.3 kW cm\(^{-2}\)) and 404 nm activation. UV-intensity was adjusted manually using an OD-density-wheel to maintain optimal fluorophore density (up to 12 W cm\(^{-2}\)). About 7,000–10,000 frames had to be recorded in widefield-illumination at a camera frame-rate of 12.5 Hz until all PamCherry1-molecules were photoconverted and -bleached. The fluorescent signal was filtered using a 610/60 bandpass filter (AHF). During PAINT-imaging of Nile Red, 10,000 frames were collected at 50 Hz camera frame-rate in HILO-mode using the same filter set (0.7 kW cm\(^{-2}\)). R6G imaging was performed under the same conditions but using 532 nm illumination and a different dichroic mirror (F58-532, AHF) and emission filter (F47-570, AHF). After all post-labeling steps,
dSTORM-imaging of Alexa Fluor 647 was performed in PBS containing 100 mM cysteamine (MEA, Sigma) pH 8.5 using the 647 nm line (2 kW cm\(^{-2}\)) for excitation and 488 nm for reactivation (up to 30 W cm\(^{-2}\)). 6,000–10,000 frames were recorded at 33 Hz. The obtained data was analyzed with rapiddSTORM [49] and resulting images were post-processed with Fiji.

4.5. sptPALM imaging and analysis

Bacteria (KF26, [20]) were grown at 32°C overnight in EZR medium with 100 µg ml\(^{-1}\) Ampicillin for selection. Cells were incubated in Edu (10 µM final concentration) for 30 min. Bacteria were then immobilized on a poly-L-lysine-coated well (Nunc Lab-Tek chamber, 8 wells) for live cell imaging in 300 µl Edu\(^{-}\)-containing EZ rich defined medium (EZ-RDM) was prepared with modifications from the original Neidhardt protocol [30] following the protocol by Blattner et al (www.genome.wisc.edu/resources/protocols/ezmedium.htm). The setup was used as described above, except that a pulsed photoactivation utilizing a pulse generator (Stanford Research Systems, DG645) was applied, with UV radiation (2.3 W cm\(^{-2}\)) of 1 ms every 500 ms. The pulsed photoactivation allows to keep the stochastically activated molecules well separated over time during the trajectory acquisition and avoid undesired track merging. Tracking data were collected imaging RNAP-PAmCherry1 with 0.9 kW cm\(^{-2}\) of 568 nm readout and merging. Tracking data were collected imaging RNAPs within the MetaMorph software environment analyzed using custom software written as a plug-in segmentation [51] and simulated annealing [52, 53] algorithms. Molecule localization and dynamics were analyzed using custom software written as a plug-in running within the MetaMorph software environment (Molecular Devices). We evaluated and visualized only trajectories with a minimum trajectory length of 80 ms (4 frames) up to 10 s (500 frames).

After labeling, cells were covered with a buffer solution (0.1 M MEA in PBS, pH 8.5) and dSTORM-imaging of Alexa Fluor 647 was performed at 50 Hz using the 647 nm line (5 kW cm\(^{-2}\)). Data analysis and super-resolution image reconstruction was performed using rapiddSTORM. The resulting images were post-processed and overlaid with the RNAPs trajectories using Fiji.

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