Micro**Commentary**

Extending the tools of single-molecule fluorescence imaging to problems in microbiology

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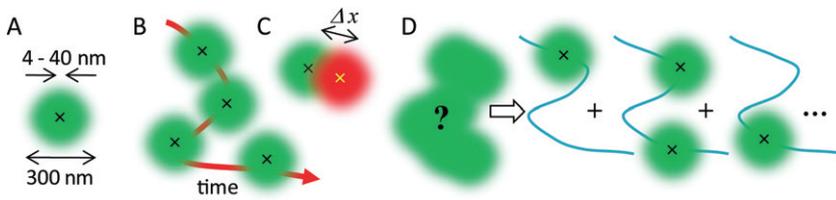
Summary

Single-molecule fluorescence microscopy enables non-invasive, high-sensitivity, high-resolution imaging, and this direct, quantitative method has recently been extended to understanding organization, dynamics and cooperativity of macromolecules in prokaryotes. In this issue of *Molecular Microbiology*, Bakshi *et al.* (2012) examine fluorescently labelled ribosomes and RNA polymerase (RNAP) in live *Escherichia coli* cells. By localizing individual molecules with 30 nm scale accuracy, they resolve the spatial distribution of RNAP (and thus of the *E. coli* nucleoid) and of the ribosomes, measure diffusion rates, and sensitively count protein copy numbers. This work represents an exciting achievement in terms of applying biophysical methods to live cells and quantitatively answering important questions in physiologically relevant conditions. In particular, the authors directly relate the positions, dynamics, and numbers of ribosomes and RNAP to transcription and translation in *E. coli*. The results indicate that, since the ribosomes and the nucleoid are well segregated, translation and transcription must be predominantly uncoupled. As well, the radial extension of ribosomes and RNAP to the cytoplasmic membrane is consistent with the hypothesis of transertion (simultaneous insertion of membrane proteins upon translation).

Fluorescence microscopy is traditionally limited in its resolution to at best 200 nm by the diffraction limit of light. For investigations of subcellular structures of bacterial cells, use of this optical technique is therefore limited, and as a result, an extensive description of localization and dynamics in prokaryotes is not yet available. Other techniques,

like electron microscopy, do achieve nanometre-scale resolution, but biological applications of these methods are generally limited to fixed or frozen cells, processes which can introduce artefacts and certainly preclude studies of dynamics. Fluorescence microscopy, on the other hand, is non-invasive and compatible with live-cell imaging. Furthermore, many bright and specific labels have been developed for fluorescence imaging, permitting accurate identification of molecules of interest. An important development in optical microscopy has therefore been the advent of single-molecule fluorescence (SMF) imaging (Moerner and Kador, 1989). In addition to providing enough sensitivity to visualize even low copy number proteins without overexpression, and to enabling heterogeneous behaviours to be untangled, SMF brings the advantages of fluorescence to the high-resolution regime: by visualizing individual, isolated fluorescent molecules and fitting their emission pattern (Betzig, 1995), SMF enables live-cell imaging with nanometre resolution in a conventional fluorescence microscope.

The ability to super-localize an emitter with SMF is used in four main ways: for localization, tracking, determination of relative positions and super-resolution imaging (Fig. 1). By imaging isolated single molecules, the emitter location, which corresponds to the centre of the emission pattern, can be pinpointed (Fig. 1A). Repeating such measurements while acquiring a movie permits the single molecule to be tracked over time (Yildiz *et al.*, 2003), and from this, the speed and trajectory, or the diffusion coefficient in the case of Brownian motion, are obtained (Fig. 1B). Although SMF imaging requires fluorescent molecules to be well separated, Fig. 1C shows that two closely neighbouring molecules can still be distinguished, and therefore colocalized, if their emission (or excitation) wavelengths are different (Churchman *et al.*, 2005). Finally, even molecules with the same colour can be distinguished if they switch from an emissive ('bright') to a non-emissive ('dark') state through process like photoswitching, blinking or absorption/desorption (Betzig *et al.*, 2006; Hess *et al.*, 2006; Rust *et al.*, 2006; Sharonov and Hochrasser, 2006; Vogelsang *et al.*, 2009). As depicted in Fig. 1D, only a small subset of emitters are in the 'bright' state in any

given imaging frame, such that the observed emission does not overlap. The molecules are localized and their positions recorded in every imaging frame, and over time, a map of localizations is reconstructed, giving rise to a super-resolution image.

Single-molecule fluorescence imaging methods have been developed and proven over the past 20 years, and SMF is now ripe for application to real biological problems. In particular, live-cell SMF has recently begun to address important questions in prokaryotic biology. For example, the high sensitivity of SMF has been used in *Escherichia coli* to identify low-level gene expression (Yu *et al.*, 2006), probe transcription factor dynamics (Elf *et al.*, 2007), and examine the stochasticity of phenotype switching (Choi *et al.*, 2008). Single macromolecules have been tracked to characterize the motion of mRNA in *E. coli* (Golding and Cox, 2006) and proteins in *Caulobacter crescentus* (Deich *et al.*, 2004; Kim *et al.*, 2006; Bowman *et al.*, 2008). Single-molecule super-resolution reconstructions have been used to deduce the superstructure of assemblies of MreB in *C. crescentus* (Biteen *et al.*, 2008) and FtsZ in *E. coli* (Fu *et al.*, 2010) and novel SMF techniques have uncovered in three dimensions the distributions of nucleoid-associated proteins in *E. coli* (Wang *et al.*, 2011), and the structures of crescentin (Lew *et al.*, 2011) and the FtsZ Z-ring (Biteen *et al.*, 2012) in *C. crescentus*.

In the present paper, SMF is used to directly relate the localization, dynamics and numbers of ribosomes and RNA polymerase (RNAP) molecules to transcription and translation in live *E. coli* cells. Based on two strains of cells, one in which the ribosomal S2 protein is genetically labelled with the fluorescent protein (FP) YFP, and one in which the RNAP β' subunit is fused to yGFP, the ability of Bakshi *et al.* to detect and localize single copies of these proteins enables three types of measurements: (i) ribosome and RNAP positions are determined with high accuracy, (ii) the motion of individual ribosomes is directly observed and their diffusion coefficient obtained and (iii) protein copy numbers and intracellular concentrations are quantified. These experiments are enabled by the reversible bleaching of the FP labels (Biteen *et al.*, 2008), which permits individual molecules within an ensemble to be observed one by one. The total number of labelled molecules is determined from the ratio of the initial fluorescence intensity before photobleaching to the average measured intensity of single, isolated molecules observed

after bleaching and spontaneous return to the 'bright' state. The authors determine that there are on average 4600 RNAPs and 55 000 ribosomes per cell. Interestingly, based on the spatial distribution of the ribosomes, it is shown that the ribosome-rich endcap regions of the cell are 22 % ribosomes by volume. This number illustrates the extent of macromolecular crowding inside the cell, and motivates live-cell experiments: an accurate characterization of biological processes cannot be obtained in the absence of this crowding and heterogeneity, which affect molecular interactions, diffusion rates and competition for binding sites.

Beyond a proof-of-principle experiment, by probing both RNAP and ribosome localization and dynamics, Bakshi *et al.* have set up a system where they can quantitatively infer the spatiotemporal relation, if any, between translation and transcription. Several important conclusions are drawn from the current work. First, the model of co-transcriptional translation (Woldringh, 2002), in which ribosomes are translating mRNA strands as they are being synthesized by RNAP, is examined. Based on the observation here of a very high degree of segregation of ribosomes and RNAP, as well as the fact that RNAP is excluded entirely from the ribosome-rich endcaps of the cell, it seems that at least in *E. coli*, co-transcriptional translation is highly unlikely to be dominant. Indeed, the authors show that the majority of ribosomes are some 300–500 nm away from DNA. This is consistent with the known timescales of transcription of a typical protein and degradation of the message. These conclusions are consistent with prior findings for *Bacillus subtilis* (Lewis *et al.*, 2000; Mascarenhas *et al.*, 2001), but disagree with observations in *C. crescentus* (Llopis *et al.*, 2010). This discrepancy remains to be explored; while *C. crescentus* does have some unique protein localization characteristics due to its asymmetric division, direct comparison studies between these different Gram-negative species would be beneficial.

Another open question in prokaryotic biology is what, if anything, is the role of transertion, the insertion of membrane proteins directly at the time of expression (Woldringh, 2002)? Consistent with the transertion model, Bakshi *et al.* find that significant quantities of both ribosomes and RNAPs extend out to the inner membrane. Additionally, arresting translation with chloramphenicol shrinks the nucleoid, perhaps due to dissociation of ribo-

somes from the membrane. It is interesting to note that their data indicate that while transertion should be possible on the radial edges of the cells, RNAP is excluded from cell endcaps, making transertion unfeasible at the poles. The possibility of transertion could potentially be studied by SMF investigations of specific labelled mRNAs in order to differentiate between the locations of those encoding membrane proteins and those encoding cytoplasmic proteins. It would also be interesting to extend the model to periplasmic and outer-membrane proteins.

Overall, the application of physical techniques to real problems in microbiology requires excellent communication between physical and natural scientists, as well as the evolution of meaningful, long-term collaborations between biologists and biophysicists. It is important that we begin to speak one another's language and educate each other about the important challenges and open questions in our respective fields. Such discourse will motivate the development of innovative new methods to answer relevant questions. As well, an open dialogue between physical and natural scientists will prevent the misinterpretation of data. Every method, whether biochemical or biophysical, must be evaluated for its biases and artefacts, and careful controls must be performed. The best work will come from collaborative efforts combining modern genetics, imaging and modelling.

We expect to see a dramatic increase in the number of microbiologists using SMF as this technology continues to become more accessible. Still, some important questions cannot be answered with the current state of the art, and new methods are being developed for SMF and adapted for live bacterial cell imaging. One important challenge lies in the need to infer information about a three-dimensional (3D) cell from a two-dimensional measurement. Continued development of techniques for 3D SMF tracking and imaging is therefore very important. Methods to extend SMF imaging to 3D include astigmatism (Huang *et al.*, 2008), multiplane methods (Juette *et al.*, 2008), optical sectioning (Fölling *et al.*, 2007), interferometry (Shtengel *et al.*, 2009) and double-helix point-spread function microscopy (Pavani *et al.*, 2009). Interestingly, 3D superresolution images of protein superstructures in live *C. crescentus* cells have recently been achieved with a double-helix point spread function microscope (Lew *et al.*, 2011) and an astigmatic lens (Biteen *et al.*, 2012).

Another challenge for SMF is its finite acquisition speed. Considering that it takes thousands of images to build up a super-resolution reconstruction, the timescale of these experiments is generally on the order of many seconds to a few minutes. Although single-particle tracking is a faster technique, requiring only ~ 10–20 imaging frames, getting reliable statistics requires many trajectories to be mapped consecutively in the same cell (Manley *et al.*, 2008). Many structural features and colocalization events are not static for such long times, and therefore cannot be probed. Moreover, Bakshi *et al.* mention that, although the overall nucleoid structure evolves slowly, the nucleoid edges, where RNAP and ribosomes could potentially interface, are likely to be dynamic, and so the extent of mixing is not evaluated precisely in the current work. We certainly expect a move towards faster imaging modalities in the future.

A final limitation of SMF imaging is the brightness of the probes. Spatial accuracy is proportional to fluorescent label brightness, and the accuracy of calculated diffusion coefficients depends on the length of measured trajectories. Brighter, longer lived fluorescent labels are therefore sought to improve the signal to noise ratio. This can be achieved via several routes, including engineering better FPs (Shaner *et al.*, 2008), improving techniques for highly specific, intracellular labelling of intracellular targets with organic fluorophores (Wombacher and Cornish, 2011), and developing generalizable platforms for enhancing emission from existing labels, for instance by plasmonenhanced fluorescence (Lakowicz, 2001). Overall, as methods are improved and adapted for the problems of microbiology, we can anticipate gaining a richer, more dynamic picture of the inner workings of bacteria.

Acknowledgements

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