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Systems biophysics: Single-molecule optical proteomics in single living cells

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Abstract

How does the interplay between biomolecules result in the emergence of cellular complexity at higher length scales? This interplay in even simple biological processes is often too challenging to probe using traditional experimental tools of ensemble averaging across several thousands of molecules. Instead, insight can be gained using single-molecule techniques which can unpick the heterogeneity in physical/chemical properties of biomolecules and their cellular interactions. Significant understanding of many biological systems can be gained using techniques which apply advanced fluorescence microscopy to determine the cellular localization, dynamics and interaction kinetics of single functional proteins, whilst retaining the native context of live cells. Here, we report recent advances applied to cell motility, DNA replication and gene regulation in model unicellular organisms.

Highlights

- Single-molecule optical proteomics can probe whole biological systems
- Several processes (motility, DNA replication, gene regulation) have been probed
- Real-time readout of molecular systems can be probed in model unicellular organisms

Introduction

Systems biology grew from seminal studies of 19th century physiologist Claude Bernard, developing homeostasis concepts: an organism's internal environment is regulated to optimize viability (1). This regulation involves interactions between multiple systems acting over multiple length and time scales. But what is the correct level at which to understand biology? Reductionists speculate we can understand life from knowledge of the individual molecules present. This notion is partially correct that it is not only molecules that are important, but also how they interact. Integrationist approaches have value, physicists/mathematicians know this well from emergent behaviours in non-biological systems: these are difficult to predict from raw composition alone. As to where to draw the line regarding the best scale to understand biology, this is a matter of ongoing debate (2) better suited for philosophers.

Every organism is semi-arbitrarily sub-divided into 'functional units' – organs, cells, molecules, coordinated into one 'functional system'. Whether it is a multicellular organism, e.g. a human body, or a single cell, e.g. yeast, it is not sufficient to study individual components alone to understand the activity of the entire system. Fuller insights are achieved if as many interactions as possible are considered. Systems biology uses approaches from engineering to address this challenge: combining

experimental and mathematical/computational tools to model networks of interacting elements. However, traditional methods struggle to investigate processes on molecular scales. Single-molecule cellular biophysics (3) is emerging as an invaluable tool to study living systems in their physiological context. Such approaches have illuminated processes that were previously not possible due to technological limitations, like bacterial cell motility, protein folding/movement, DNA architecture and replication (4, 5).

Much of systems biology has adopted computational aspects to model biological processes. But it is only in the past decade that these tools have been coupled to advanced biophysical techniques to more precisely measure molecular parameters which can be used in these models. A challenge today lies in matching the exquisite quality of modelling to the complex nature of biophysics-derived experimental data. Their coupling results in systems biophysics. Systems biophysics has potential to bridge the genotype to phenotype gap (6); we have a good understanding of composition, type and numbers of genes from sequencing and also can quantify phenotypes. Nevertheless, it is hard to correlate these using traditional experimental approaches.

Proteomics, a phrase first coined in 1997 to describe the study of the composition and interactions of the complete set of proteins in an organism (7), i.e. the proteome, grew from initial in vitro biochemical methods culminating in advanced co-fractionation and mass spectrometry methods to analysing network of interacting protein containing several hundred different proteins (8–11), including associated computational tools which use correlation analysis from these data to determine putative interaction interfaces for protein-protein interactions (12). Useful associated proteomics resources in particular now exist for the genomics cancer biology resource of the cancer biology genome atlas, to indicate levels of functional protein expression for different cancer genes (13). More recent methods tools have used ‘structural proteomics’ techniques, in particular higher throughput methods of X-ray crystallography, to yield insights into the structure-function relations across protein networks within the proteome (14), and more recently including methods of cryo-electron microscopy to visualize a range of high molecular weight protein complexes with a view to establishing a ‘visual proteomics’ approach to quantify macromolecular interactions (15).

Optical spectroscopy methods have been used to fingerprint peptides by employing infrared spectroscopy methods (16), however, significant advantages are made possible by instead using visible light microscopy methods which can retain the physiological context of the cell of tissue. The general use of a range of advanced optical imaging techniques to quantify protein networks, typically in both cells and tissues, is termed ‘optical proteomics’, and has been used to probe several complex protein networks, including those involved in cancer formation (17). Recent developments have enabled high throughput methods to analyse single cells using optical proteomics methods using flow

cytometry tools (18). Systems biophysics can use, in particular, single-molecule fluorescence microscopy to track individual protein molecules in living cells. Novel light microscopy combined with genetics methods now enable real-time observations of molecular exchange/turnover in functioning systems of several model unicellular organisms. This 'single-molecule optical proteomics' has been applied to cell motility, chemotaxis, bioenergetics, signalling, DNA replication, and gene regulation. The experimental approaches often use fluorescent proteins to pinpoint native proteins in a cell, with laser illumination, beam-shaping, super-resolution microscopy and novel image analysis algorithms dedicated to extracting tiny signals from the noisy 'soft matter' environment (19, 20).

Here we report recent advances of single-molecule optical proteomics in unicellular organisms, enabling insight at 'bottom-up' molecular scales, and associated developments required for the new biophysical technology which, in itself, can be designed using systems engineering principles informed by underlying biological processes (21).

Main text

Traditional quantification methods for the amount of proteins in cells involve ensemble average analysis of populations, whereas, single-molecule biophysics techniques offer experimental and theoretical tools that use physics to understand life at the molecular level (22). Focusing on biomolecules as the minimal functional unit, single-molecule biophysics impacts various fields, including medical immunology and synthetic/systems biology, by enhancing spatial and temporal resolution of experimental data (4). In particular, 'single-molecule cell biology' is emerging as its own discipline (23), enabling cell biology studies using advanced light microscopy (24) with unprecedented sensitivity (25), including rendering 3D spatial information of protein superstructures to super-resolution precision from single functional cells (26). Modern techniques permit the study of complex cellular processes such as signal transduction directly (27), allowing more precise insight based on molecular stoichiometry, mobility, copy numbers, and localization within cells (**Figure 1**). A principle technique used is fluorescence microscopy, which provides a reasonable signal-to-noise ratio for detection with relatively small perturbation of native physiology compared to many biophysical approaches. Several analytical methods can now extract meaningful information from these measurements (28, 29). Genomically integrated fusions of fluorescent proteins with native proteins enable 100% tagging efficiency and similar levels of protein expression to untagged strains. Organic dyes are also used in single-molecule imaging, brighter and more photostable than fluorescent proteins, but not genetically encodable which limits their labelling specificity (30). A variety of protein labels and the microscopy techniques developed, have been reviewed recently (31).

The combination of advanced light microscopy with genetics tools enables enormous insights into functional behaviours of even low copy number proteins (32) in unicellular organisms or single cells (33). Different studies have used single-molecule/cell and super-resolution microscopy methods on integrated membrane proteins (34, 35), including interaction networks like oxidative phosphorylation (36–40), cell division (41, 42) and protein translocation (43), with several insights into bacterial cell motility (44–47). More recently, studies look inside cells as opposed to on their surfaces, including DNA replication/remodelling/repair (48–50), and processes relevant to biomedicine, like bacterial infection (51–53).

Flagellar motors in bacteria

The bacterial flagellar motor is an exemplar complex molecular machine, ~50nm in diameter comprising ~13 different core proteins (54). One of the first single-molecule optical proteomics studies used total internal reflection fluorescence (TIRF) (**Figure 2A**), a 'nearfield' approach which delimits laser excitation to ~100nm from a microscope coverslip/slide surface (30), enabling enhancements in contrast for labelled components in cell membranes. Here, Escherichia coli bacteria were modified to label flagellar motors, specifically a force-generating protein MotB with green fluorescent protein (GFP) (**Figure 3A**) (55). The lateral optical resolution was limited by the diffraction of light to ~200-300nm, but TIRF enhances the axial resolution by delimiting laser excitation to the surface (56). TIRF is used in many surface-related questions of cell biology, enabling the study of molecules and structures integrated into cell membranes. The fact that GFP photobleaches in a step-wise manner (**Figure 2B**) allows estimation of the number of GFP present in a motor, suggesting ~22 MotB molecules on average in each but with real variability about this mean. Molecular variability is important in maximising robustness of a cellular response against microenvironmental changes, however, it is challenging to experimentally measure cell-by-cell using other techniques.

The authors also observed a freely diffusing membrane pool of MotB. Molecular turnover of MotB could be observed between this pool and motors by using fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) (**Figure 2C**). These techniques can be applied to other systems to determine molecular mobility as well as kinetics parameters (57). Further applications of these techniques showed two populations of a protein FliM, which functions as a part of a complex associated with the flagellar motor to control direction of rotation. One FliM component was tightly connected to the motor whilst another underwent turnover dependent on the chemotaxis signalling protein CheY (58). Thus, single-molecule fluorescence microscopy revealed direct dynamic regulation of functioning molecular machines. Further studies of E. coli flagellar motors employing

gold and polystyrene beads uncovered the role of Na^+ ions in kinetic parameters of the motor rotation mechanisms (59).

DNA studies (replication, remodelling, and repair)

Single-molecule optical proteomics has enabled insight into DNA and associated protein complexes. DNA replication involves molecular machinery comprising over 11 different proteins interacting in concert with each other and DNA (60). Recent work on DNA replication in E.coli indicated both leading and lagging strand synthesis is a discontinuous mechanism undergoing constant interruption. These studies were enabled by single particle tracking software which pinpointed the location of fluorescently tagged components with a few tens of nm precision (61–64). These tracking tools have benefited from 'step detection' algorithms developed from mechanical experiments (65–68). Similarly, computational methods infer the 'mode' of diffusion, e.g. whether a protein is diffusing in the cytoplasm or actually bound at its point of action to the DNA (69). Using these analysis tools the DNA polymerase III holoenzyme (PolIII), the primary enzyme complex involved in DNA synthesis in prokaryotes, was shown to frequently dissociate from the replisome and exchange with free copies diffusing in the cytoplasm. In contrast, DnaB, a replicative helicase which unravels DNA prior to template copying, stays attached to the replication fork, providing an anchor for replisome assembly (70). A similar study on PolIII has suggested a concentration dependent exchange mechanism which could provide replisome plasticity and stability at the same time (71).

Observations have also been made using Photoactivated Localization Microscopy (PALM) (**Figure 2D**), utilising stochastic fluorophore activation/imaging in multiple cycles. Only a few fluorophores are excited per cycle so there is minimal overlap of diffraction-limited images generated from each dye (72). Another study later confirmed the discontinuous behaviour of replisome components (73) using TIRF to image single replisomes in vitro. However, the DNA synthesis efficiency was far lower than in vivo, demonstrating the attraction of these single-molecule experiments in living cells.

A valuable optical proteomics method to study interactions is Förster resonance energy transfer (FRET) (**Figure 2E**). FRET utilises non-radiative energy transfer between a donor and acceptor molecule, which are often both fluorescent. If these molecules are within a few nm of each other an excited donor can transfer energy to an acceptor through resonance of overlapping molecular orbitals (74). FRET has been used in monitoring opening/closing of budding yeast Saccharomyces cerevisiae ring-shaped helicases Mcm2-7 upon replication initiation (75), DNA unwinding by Werner syndrome ATP-dependent helicase (76), and various molecular studies on DNA origami structures (77).

A related technique to PALM is stochastic optical reconstruction microscopy (STORM), in which fluorophores are photoswitched (78). STORM has been implemented in genomics studies to detect multiple mRNA species on a single S. cerevisiae cells. The strategy used single-molecule fluorescent in situ hybridization (FISH) with spectral 'barcoding', managing to profile 32 calcium stress-response genes. Using STORM for visualisation it was possible to calculate mRNA abundancies for each transcript with/without calcium (79). Single-molecule FISH on bacterial transcription showed that transcriptional bursting depends on the concentration of gyrase within the cell (80) but also interplay between RNA polymerases on DNA (81).

Gene regulation in single cells observed on a molecular scale

Living organisms respond to environmental changes by adjusting their 'life style'. Stimuli are received/detected by receptors and transmitted via chemical cascades through the cytoplasm leading to cellular responses (**Figure 3B**). On the molecular scale, responses involve changes to gene expression at transcriptional/translational levels. A key component in gene regulation is a transcription factor: a protein which binds to promoter regions of target genes to control expression. In a study on mammalian c-Myc and P-TEFb, Izeddin et al, suggest that these transcription factors explores the nucleus in two different manners which determine the speed and the distance they can travel in order to find their targets (82). Two-colour single-molecule imaging revealed different modes of DNA binding of glucocorticoid receptor in mammalian cells (83).

The glucose repression pathway in S. cerevisiae is a model system for studying signal transduction – yeast is easy to grow and genetically modify, and it is simple to alter glucose concentrations in the media to observe changes to appropriate transcription factors. Studies to probe this pathway directly using single-molecule optical proteomics in live cells were published recently (84–86). Researchers tracked a GFP-labelled repressor Mig1, a transcription factor which regulates metabolism of non-glucose carbon sources, such as sucrose, maltose, galactose, and responses to glucose starvation, by changing its phosphorylation status and cellular localization. Slimfield, a microscopy method using delimited illumination volumes for excitation of a single cell, enabled rapid imaging on a millisecond time scale (27, 87). With this technique Mig1 copy numbers were determined cell-by-cell, as well as changes in its distribution in different subcellular compartments, utilising automated image segmentation (88). The researchers also used STORM to determine dynamic Mig1 behaviour, including diffusion and stoichiometry, to shed light on how transcription factors find their targets (86). The results revealed that Mig1 forms oligomers whose mobility depends on extracellular glucose, suggesting that a transcription factor cluster is the functional unit of gene regulation. Similar

clusters have been previously observed by PALM in studies on RNA polymerase II (89) suggesting importance of protein oligomerization in transcription regulation.

Conclusions and future perspectives

Single-cell/molecule studies give us precise and quantitative information about biological systems. Fluorescent reporters are the most frequently used tags, however, they have poor photostability which limits observations. Also, they are as large as a native protein under investigation and so may disturb some physiological functions. These issues have driven attempts to develop label-free techniques. For example, digital holographic imaging (90, 91) has been applied to malaria parasites and revealed new structural details of flagella morphology (92). Philip Kukura et al. has developed an interferometric label-free scattering microscope (iSCAT) (93, 94) which has been used in studies of single motor proteins dynamics (95), enabling imaging of microtubule disassembly (96) and revealing mechanistic insights into myosin 5 (97) and kinesin-1 (98).

Every functional unit of life, such as a molecule/cell, exists in an environment containing other such units across multiple length and time scales. Depending on their role, single molecules/cells have multiple states which determine aspects of molecular/cellular communication. Single-molecule optical proteomics, in combination with other methods, may have future utility for probing multicellular samples, such as bacterial biofilms and tissues. For example, single-molecule force spectroscopy has been used to study cell-cell adhesion forces in Staphylococcus aureus biofilms focusing on SdrC, an important surface protein (99). It is now possible to quantify single mRNA molecules in mammalian tissues by using a combination of single fluorophore-labelled short nucleotides hybridized to target mRNA (100–102). Another technique, a digital proximity ligation assay for absolute mRNA and protein quantification applied on single mammalian cells has been reported recently showing that mRNA amounts do not equate to actual protein produced (103). The visualisation of mRNA in live yeast cells is also possible via binding of fluorescently labelled coat proteins to the stem loop repeats introduced into gene of interest. Due to binding specificity, such technique allows studying two RNAs within the same cell simultaneously (104, 105). Single-molecule in situ hybridization was also used for post-transcriptional quantification in Drosophila brains (106), enabling visualisation of structures including neuronal stem cells and mushroom body neuropils. Extracellular space organisation of rat brains was studied by tracking near-infrared fluorescence from single-wall carbon nanotubes injected intraventricularly (107). Nevertheless, it is apparent that experiments on cell populations are not yet close enough to native physiological conditions, for example leading to higher drug resistance of cells in a 2D sample as opposed to standard conditions in vivo (108). To combat these problems, the development of new organ-on-a-chip technologies (109) combined with super-resolution microscopy

has a potential to increase our understanding of molecular/cellular functions on an organ level, thus aiding 'smart' drug development personalized to individual patients.

Emerging developments drive optical techniques to higher levels of precision and physiological relevance to enable faster real-time, molecular in vivo imaging of several different proteins in interacting biological systems. The result may enable the establishment and validation of far more realistic mathematical/computational models of protein networks which are accurate down to the molecular level. These new approaches may push forward mechanistic understanding of the most complex processes that comprise life as we know it.

Figure legends:

Figure 1. Summary of the types of data which are possible to obtain using single-molecule optical proteomics techniques.

Figure 2. (**A**) Schematic representation of the TIRF imaging technique. (**B**) Step-wise photobleaching of GFP molecules. Schematic representations of (**C**) FLIP and FRAP, (**D**) PALM and (**E**) FRET techniques.

Figure 3. (**A**) Schematic representation of the E.coli flagellar motor structure (left panel), and TIRF images of MotB-GFP proteins associated with a motor before and after photobleaching (right panel). (**B**) A simplified scheme of signal transduction leading to a response on a gene regulation level (left panel). An example of Mig1-GFP protein localization within the yeast S. cerevisiae under high and low glucose conditions.

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References:

Special interest (•) or outstanding interest (••)

1. Leake MC (2016) *Biophysics : tools and techniques* (CRC Press).
2. Noble D (2006) *The music of life : biology* beyond the genome (Oxford University Press).
3. Leake MC (2013) Single-molecule cellular biophysics (Cambridge University Press).
4. Leake MC (2013) The physics of life: one molecule at a time. Philos Trans R Soc Lond B Biol Sci. doi:10.1098/rstb.2012.0248.
5. Deniz AA, Mukhopadhyay S, Lemke EA (2008) Single-molecule biophysics: at the interface of biology, physics and chemistry. J R Soc Interface 5(18).
6. Gjuvsland AB, Vik JO, Beard DA, Hunter PJ, Omholt SW (2013) Bridging the genotype-phenotype gap: what does it take? J Physiol 591(8):2055–66.
7. James P (1997) Protein identification in the post-genome era: the rapid rise of proteomics. Q Rev Biophys 30(4):279–331.
8. Larance M, Lamond AI (2015) Multidimensional proteomics for cell biology. Nat Rev Mol Cell Biol 16(5):269–280.
9. Bantscheff M, Schirle M, Sweetman G, Rick J, Kuster B (2007) Quantitative mass spectrometry in proteomics: a critical review. Anal Bioanal Chem 389(4):1017–1031.
10. Bensimon A, Heck AJR, Aebersold R (2012) Mass Spectrometry–Based Proteomics and Network Biology. Annu Rev Biochem 81(1):379–405.
11. Sabidó E, Selevsek N, Aebersold R (2012) Mass spectrometry-based proteomics for systems biology. Curr Opin Biotechnol 23(4):591–597.
12. Drew K, Müller CL, Bonneau R, Marcotte EM (2017) Identifying direct contacts between protein complex subunits from their conditional dependence in proteomics datasets. PLOS Comput Biol 13(10):e1005625.
13. Li J, Lu Y, Akbani R, Ju Z, Roebuck PL, Liu W, Yang J-Y, Broom BM, Verhaak RGW, Kane DW, Wakefield C, Weinstein JN, Mills GB, Liang H (2013) TCPA: a resource for cancer functional proteomics data. Nat Methods 10(11):1046–7.
14. Manjasetty BA, Büssow K, Panjikar S, Turnbull AP (2012) Current methods in structural proteomics and its applications in biological sciences. 3 Biotech 2(2):89–113.
15. Nickell S, Kofler C, Leis AP, Baumeister W (2006) A visual approach to proteomics. Nat Rev Mol Cell Biol 7(3):225–230.
16. Fournier F, Gardner EM, Guo R, Donaldson PM, Barter LMC, Palmer DJ, Barnett CJ, Willison KR, Gould IR, Klug DR (2008) Optical fingerprinting of peptides using two-dimensional infrared spectroscopy: Proof of principle. Anal Biochem 374(2):358–365.
17. Kelleher MT, Fruhwirth G, Patel G, Ofo E, Festy F, Barber PR, Ameer-Beg SM, Vojnovic B, Gillett C, Coolen A, Kéri G, Ellis PA, Ng T (2009) The potential of optical proteomic technologies to individualize prognosis and guide rational treatment for cancer patients. Target Oncol 4(3):235–52.

18. Etcheverry S, Faridi A, Ramachandraiah H, Kumar T, Margulis W, Laurell F, Russom A (2017) High performance micro-flow cytometer based on optical fibres. Sci Rep 7(1):5628.

19. Dobbie IM, Robson A, Delalez N, Leake MC (2009) Visualizing single molecular complexes in vivo using advanced fluorescence microscopy. J Vis Exp (31):1508.

20. Harriman, O L J, Leake MC (2011) Single molecule experimentation in biological physics: exploring the living component of soft condensed matter one molecule at a time. J Phys Condens Matter.

21. Harriman, O L J, Leake MC (2013) A system-level approach to single-molecule live-cell fluorescence microscopy. Infocus 30:5–18.

••22. Miller H, Zhou Z, Shepherd J, Wollman A, Leake M (2017) Single-molecule techniques in biophysics: a review of the progress in methods and applications. Reports Prog Phys. doi:10.1088/1361-6633/aa8a02.

This is a modern and comprehensive review of the recent progress made in single-molecule biophysics techniques.

23. Leake MC (2010) Shining the spotlight on functional molecular complexes: The new science of single-molecule cell biology. Commun Integr Biol 3(5):415–8.

24. Wollman AJM, Nudd R, Hedlund EG, Leake MC (2015) From Animaculum to single molecules: 300 years of the light microscope. Open Biol 5(4):150019–150019.

25. Lenn T, Leake MC (2012) Experimental approaches for addressing fundamental biological questions in living, functioning cells with single molecule precision. Open Biol 2(6):120090.

26. Lew MD, Lee SF, Ptacin JL, Lee MK, Twieg RJ, Shapiro L, Moerner WE (2011) Three-dimensional superresolution colocalization of intracellular protein superstructures and the cell surface in live Caulobacter crescentus. Proc Natl Acad Sci 108(46):E1102–E1110.

27. Wollman AJM, Leake MC (2016) Single molecule narrowfield microscopy of protein-DNA binding dynamics in glucose signal transduction of live yeast cells. Methods Mol Biol 1431:5–15.

28. Leake MC (2014) Analytical tools for single-molecule fluorescence imaging in cellulo. Phys Chem Chem Phys 16(25):12635–47.

29. Ulbrich MH, Isacoff EY (2007) Subunit counting in membrane-bound proteins. Nat Methods 4(4):319.

30. Chiu S-W, Leake MC (2011) Functioning Nanomachines Seen in Real-Time in Living Bacteria Using Single-Molecule and Super-Resolution Fluorescence Imaging. Int J Mol Sci 12(12):2518–2542.

31. Shashkova S, Leake MC (2017) Single-molecule fluorescence microscopy review: shedding new light on old problems. Biosci Rep 37(4).

32. Huang B, Wu H, Bhaya D, Grossman A, Granier S, Kobilka BK, Zare RN (2007) Counting Low-Copy Number Proteins in a Single Cell. Science (80-) 315(5808).

33. Wu M, Singh AK (2012) Single-cell protein analysis. Curr Opin Biotechnol 23(1):83–8.

34. Bryan SJ, Burroughs NJ, Shevela D, Yu J, Rupprecht E, Liu L-N, Mastroianni G, Xue Q, Llorente-Garcia I, Leake MC, Eichacker LA, Schneider D, Nixon PJ, Mullineaux CW (2014) Localisation and interactions of the Vipp1 protein in cyanobacteria. Mol Microbiol 94(5):1179–1195.

35. Nenninger A, Mastroianni G, Robson A, Lenn T, Xue Q, Leake MC, Mullineaux CW (2014) Independent mobility of proteins and lipids in the plasma membrane of Escherichia coli. Mol Microbiol 92(5):1142–53.

36. Lenn T, Leake MC (2016) Single-molecule studies of the dynamics and interactions of bacterial OXPHOS complexes. Biochim Biophys Acta - Bioenerg 1857(3):224–231.

37. Llorente-Garcia I, Lenn T, Erhardt H, Harriman OL, Liu L-N, Robson A, Chiu S-W, Matthews S, Willis NJ, Bray CD, Lee S-H, Shin JY, Bustamante C, Liphardt J, Friedrich T, Mullineaux CW, Leake MC (2014) Single-molecule in vivo imaging of bacterial respiratory complexes indicates delocalized oxidative phosphorylation. Biochim Biophys Acta. doi:10.1016/j.bbabio.2014.01.020.

38. Lenn YT, Leake MC, Mullineaux CW (2008) Are Escherichia coli OXPHOS complexes concentrated in specialized zones within the plasma membrane? Biochem Soc Trans 36(Pt 5):1032–6.

39. Lenn T, Leake MC, Mullineaux CW (2008) Clustering and dynamics of cytochrome bd-I complexes in the Escherichia coli plasma membrane in vivo. Mol Microbiol 70(6):1397–407.

40. Badrinarayanan A, Leake MC (2016) Using Fluorescence Recovery After Photobleaching (FRAP) to Study Dynamics of the Structural Maintenance of Chromosome (SMC) Complex In Vivo. Methods Mol Biol 1431:37–46.

41. Chiu S-W, Roberts MAJ, Leake MC, Armitage JP (2013) Positioning of chemosensory proteins and FtsZ through the Rhodobacter sphaeroides cell cycle. Mol Microbiol 90(2):322–37.

42. Bisson-Filho AW, Hsu Y-P, Squyres GR, Kuru E, Wu F, Jukes C, Sun Y, Dekker C, Holden S, VanNieuwenhze MS, Brun Y V, Garner EC (2017) Treadmilling by FtsZ filaments drives peptidoglycan synthesis and bacterial cell division. Science 355(6326):739–743.

43. Leake MC, Greene NP, Godun RM, Granjon T, Buchanan G, Chen S, Berry RM, Palmer T, Berks BC (2008) Variable stoichiometry of the TatA component of the twin-arginine protein transport system observed by in vivo single-molecule imaging. Proc Natl Acad Sci U S A 105(40):15376–81.

44. Reid SW, Leake MC, Chandler JH, Lo CJ, Armitage JP, Berry RM (2006) The maximum number of torque-generating units in the flagellar motor of Escherichia coli is at least 11. Proc Natl Acad Sci U S A. doi:10.1073/pnas.0509932103.

•45. Sowa Y, Rowe AD, Leake MC, Yakushi T, Homma M, Ishijima A, Berry RM (2005) Direct observation of steps in rotation of the bacterial flagellar motor. Nature 437(7060):916–9.

Using single-molecule biophysics techniques, the authors demonstrate that the bacterial flagellar motor rotates in distinct nanoscale steps. This is the first study to use light microscopy techniques to directly demonstrate nanoscale stepping of the flagellar motor.

46. Pilizota T, Brown MT, Leake MC, Branch RW, Berry RM, Armitage JP (2009) A molecular brake, not a clutch, stops the Rhodobacter sphaeroides flagellar motor. Proc Natl Acad Sci U S A 106(28):11582–7.

47. Lo C-J, Leake MC, Pilizota T, Berry RM (2007) Nonequivalence of membrane voltage and ion-gradient as driving forces for the bacterial flagellar motor at low load. Biophys J 93(1):294–302.

••48. Reyes-Lamothe R, Sherratt DJ, Leake MC (2010) Stoichiometry and architecture of active DNA replication machinery in Escherichia coli. Science 328(5977):498–501.

Using advanced fluroesence microscopy, the authors show that the DNA replisome is composed of three DNA polymerases operates through a rocking-climbing mechanism. This is a the first study use millisecond Slmifiled microscopy to study a living cell at a single-molecule precise level.

••49. Badrinarayanan A, Reyes-Lamothe R, Uphoff S, Leake MC, Sherratt DJ (2012) In vivo architecture and action of bacterial structural maintenance of chromosome proteins. Science 338(6106):528–31.

Using advanced fluroesence microscopy, the authors show that the DNA remodelling complex operates through a rocking-climbing mechanism. This is a the first study to probe these functional molecular machines at a single-molecule precise level in live cells.

50. Wollman AJM, Syeda AH, McGlynn P, Leake MC (2016) Single-molecule observation of DNA replication repair pathways in E. coli. Adv Exp Med Biol 915:5–16.

51. Leake MC (2016) The Biophysics of Infection. Adv Exp Med Biol 915:1–3.

52. Miller H, Wollman AJM, Leake MC (2016) Designing a Single-Molecule Biophysics Tool for Characterising DNA Damage for Techniques that Kill Infectious Pathogens Through DNA Damage Effects. Adv Exp Med Biol 915:115–27.

53. Wollman AJM, Miller H, Foster S, Leake MC (2016) An automated image analysis framework for segmentation and division plane detection of single liveStaphylococcus aureuscells which can operate at millisecond sampling time scales using bespoke Slimfield microscopy. Phys Biol 5(3):55002.

•54. Sowa Y, Berry RM (2008) Bacterial flagellar motor. Q Rev Biophys 41(2):103–32.

This paper is a compehensive modern review of the bacterial flagallar motor.

••55. Leake MC, Chandler JH, Wadhams GH, Bai F, Berry RM, Armitage JP (2006) Stoichiometry and turnover in single, functioning membrane protein complexes. Nature 443(7109):355–8.

This paper uses single-molecule optical proteomics to probe fuctional flagallar motors. It is the first study to demonstrate dynamic molecular turnover in a functional molecular machine.

56. Schermelleh L, Heintzmann R, Leonhardt H (2010) A guide to super-resolution fluorescence microscopy. J Cell Biol 190(2).

57. Axelrod D, Koppel DE, Schlessinger J, Elson E, Webb WW (1976) Mobility measurement by analysis of fluorescence photobleaching recovery kinetics. Biophys J 16(9):1055–1069.

•58. Delalez NJ, Wadhams GH, Rosser G, Xue QA, Brown MT, Dobbie IM, Berry RM, Leake MC, Armitage JP (2010) Signal-dependent turnover of the bacterial flagellar switch protein FliM. Proc Natl Acad Sci U S A. doi:10.1073/pnas.1000284107.

This paper uses single-molecule imaging on fluorescent protein fusion constructs to probe the switching of fuctional flagallar motors. It is the first study to demonstrate the mechansiams of switching at a single-molecule process level of functional flagallar motors.

59. Lo C-J, Sowa Y, Pilizota T, Berry RM (2013) Mechanism and kinetics of a sodium-driven bacterial flagellar motor. Proc Natl Acad Sci U S A 110(28):E2544-51.

60. Harvey Lodish, Arnold Berk, S Lawrence Zipursky, Paul Matsudaira, David Baltimore and JD (2000) The DNA Replication Machinery. Molecular Cell Biology, 4th Edition (New York: W. H. Freeman).

•61. Miller H, Zhou Z, Wollman AJM, Leake MC (2015) Superresolution imaging of single DNA molecules using stochastic photoblinking of minor groove and intercalating dyes. Methods 88:81–8.

This paper uses new single-molecule trackng algorithms to generate super-resolution structures. It is the first to use these methoid to reconstruct imaging of single DNA molecules.

62. Wollman AJM, Miller H, Zhou Z, Leake MC (2015) Probing DNA interactions with proteins using a single-molecule toolbox: inside the cell, in a test tube and in a computer. Biochem Soc Trans 43(2):139–145.

63. Zhou Z, Miller H, Wollman A, Leake M (2015) Developing a New Biophysical Tool to Combine Magneto-Optical Tweezers with Super-Resolution Fluorescence Microscopy. Photonics 2(3):758–772.

64. Xue Q, Leake MC (2009) A novel multiple particle tracking algorithm for noisy in vivo data by minimal path optimization within the spatio-temporal volume. Proceedings - 2009 IEEE International Symposium on Biomedical Imaging: From Nano to Macro, ISBI 2009, pp 1158–1161.

65. Leake MC, Wilson D, Bullard B, Simmons RM (2003) The elasticity of single kettin molecules using a two-bead laser-tweezers assay. FEBS Lett 535(1–3):55–60.

66. Leake MC, Wilson D, Gautel M, Simmons RM (2004) The elasticity of single titin molecules using a two-bead optical tweezers assay. Biophys J 87(2):1112–35.

67. Linke WA, Leake MC, Grutzner A, Kruger M (2006) Mechanical properties of cardiac titin's N2B-region by single-molecule atomic force spectroscopy. J Stuctural Biol 155(2):263–72.

68. Linke WA, Leake MC (2004) Multiple sources of passive stress relaxation in muscle fibres. doi:10.1088/0031-9155/49/16/009.

69. Robson A, Burrage K, Leake MC (2013) Inferring diffusion in single live cells at the single-molecule level. Philos Trans R Soc L B Biol Sci 368(1611):20120029.

••70. Beattie TR, Kapadia N, Nicolas E, Uphoff S, Wollman AJ, Leake MC, Reyes-Lamothe R (2017) Frequent exchange of the DNA polymerase during bacterial chromosome replication. Elife 6:e21763.

This paper uses single-molecule optical proteomics on live bacteria to study the way the DNA is replicated. It is the first to demonstrate that DNA replication is discontinuous on both the lagging and the leading strands.

71. Yuan Q, Dohrmann PR, Sutton MD, McHenry CS, McHenry C, Marians K, Curmi P, Otting G, Dixon N, Alewood P, Jennings P, Dixon N, Huber T, Dixon N, Dixon N (2016) DNA Polymerase III, but Not Polymerase IV, Must Be Bound to a τ-Containing DnaX Complex to Enable Exchange into Replication Forks. J Biol Chem 291(22):11727–35.

72. Hess ST, Girirajan TPK, Mason MD (2006) Ultra-high resolution imaging by fluorescence photoactivation localization microscopy. Biophys J 91(11):4258–72.

73. Graham JE, Marians KJ, Kowalczykowski SC (2017) Independent and Stochastic Action of DNA Polymerases in the Replisome. Cell 169(7):1201–1213.e17.

74. Forster T (1946) Energiewanderung und Fluoreszenz. Naturwissenschaften 33(6):166–175.

75. Ticau S, Friedman LJ, Champasa K, Jr IRC, Gelles J, Bell SP (2017) Mechanism and timing of Mcm2-7 ring closure during DNA replication origin licensing. Nat Struct Mol Biol. doi:10.1038/nsmb.3375.

76. Wu W-Q, Hou X-M, Zhang B, Fossé P, René B, Mauffret O, Li M, Dou S-X, Xi X-G (2017) Single-molecule studies reveal reciprocating of WRN helicase core along ssDNA during DNA unwinding. Sci Rep 7:43954.

77. Birkedal V, Dong M, Golas MM, Sander B, Andersen ES, Gothelf KV, Besenbacher F, Kjems J (2011) Single molecule microscopy methods for the study of DNA origami structures. Microsc Res Tech 74(7):688–698.

••78. Rust MJ, Bates M, Zhuang X (2006) Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). Nat Methods 3(10):793–5.

This papers is the first to outline a now common super-resolution method called STORM.

79. Lubeck E, Cai L (2012) Single-cell systems biology by super-resolution imaging and combinatorial labeling. Nat Methods 9(7):743–748.

80. Chong S, Chen C, Ge H, Xie XS (2014) Mechanism of transcriptional bursting in bacteria. Cell 158(2):314–326.

81. Fujita K, Iwaki M, Yanagida T (2016) Transcriptional bursting is intrinsically caused by interplay between RNA polymerases on DNA. Nat Commun 7:13788.

82. Izeddin I, Récamier V, Bosanac L, Cissé II, Boudarene L, Dugast-Darzacq C, Proux F, Bénichou O, Voituriez R, Bensaude O, Dahan M, Darzacq X (2014) Single-molecule

tracking in live cells reveals distinct target-search strategies of transcription factors in the nucleus. Elife 3. doi:10.7554/eLife.02230.

83. Gebhardt JCM, Suter DM, Roy R, Zhao ZW, Chapman AR, Basu S, Maniatis T, Xie XS (2013) Single-molecule imaging of transcription factor binding to DNA in live mammalian cells. Nat Methods 10(5):421–6.

84. Shashkova S, Wollman AJM, Leake MC, Hohmann S (2017) The yeast Mig1 transcriptional repressor is dephosphorylated by glucose-dependent and -independent mechanisms. FEMS Microbiol Lett 364(14). doi:10.1093/femsle/fnx133.

•85. Wollman AJM, Leake MC (2015) Millisecond single-molecule localization microscopy combined with convolution analysis and automated image segmentation to determine protein concentrations in complexly structured, functional cells, one cell at a time. Faraday Discuss 184:401–24.

This papers uses single-molecule fluorescnece miroscopy on living cells to determine copy numbers. It is the first to demnstrate a method that can determine the copy numbers in separate sub-cellular compartments.

86. Wollman AJM, Shashkova S, Hedlund EG, Friemann R, Hohmann S, Leake MC (2017) Transcription factor clusters regulate genes in eukaryotic cells. Elife 6:e27451.

87. Plank M, Wadhams GH, Leake MC (2009) Millisecond timescale slimfield imaging and automated quantification of single fluorescent protein molecules for use in probing complex biological processes. Integr Biol (Camb) 1(10):602–12.

88. Xue QA, Jones NS, Leake MC (2010) A general appraoch for segmenting elongated and stubby biological objects: extending a chord lengt transform with the Radom transform. 2010 7th Ieee Int Symp Biomed Imaging From Nano To Macro:161–164.

89. Cisse II, Izeddin I, Causse SZ, Boudarene L, Senecal A, Muresan L, Dugast-Darzacq C, Hajj B, Dahan M, Darzacq X (2013) Real-Time Dynamics of RNA Polymerase II Clustering in Live Human Cells. Science (80-) 341(6146).

90. Liu C, Liu Z, Bo F, Wang Y, Zhu J (2002) Super-resolution digital holographic imaging method. Appl Phys Lett 81(17):3143–5.

91. Paturzo M, Merola F, Grilli S, De Nicola S, Finizio A, Ferraro P (2008) Super-resolution in digital holography by a two-dimensional dynamic phase grating. Opt Express 16(21):17107.

92. Wilson LG, Carter LM, Reece SE (2013) High-speed holographic microscopy of malaria parasites reveals ambidextrous flagellar waveforms. Proc Natl Acad Sci 110(47):18769–18774.

93. Ortega-Arroyo J, Kukura P (2012) Interferometric scattering microscopy (iSCAT): new frontiers in ultrafast and ultrasensitive optical microscopy. Phys Chem Chem Phys 14(45):15625.

94. Arroyo JO, Andrecka J, Spillane KM, Billington N, Takagi Y, Sellers JR, Kukura P (2014) Label-Free, All-Optical Detection, Imaging, and Tracking of a Single Protein. Nano Lett 14(4):2065–2070.

95. Andrecka J, Takagi Y, Mickolajczyk KJ, Lippert LG, Sellers JR, Hancock WO, Goldman YE, Kukura P (2016) Interferometric Scattering Microscopy for the Study of

Molecular Motors. Methods Enzymol 581:517–539.

96. Andrecka J, Ortega Arroyo J, Lewis K, Cross RA, Kukura P (2016) Label-free Imaging of Microtubules with Sub-nm Precision Using Interferometric Scattering Microscopy. Biophys J 110(1):214–217.

97. Andrecka J, Ortega Arroyo J, Takagi Y, de Wit G, Fineberg A, MacKinnon L, Young G, Sellers JR, Kukura P (2015) Structural dynamics of myosin 5 during processive motion revealed by interferometric scattering microscopy. Elife 4:e05413.

98. Mickolajczyk KJ, Deffenbaugh NC, Arroyo JO, Andrecka J, Kukura P, Hancock WO (2015) Kinetics of nucleotide-dependent structural transitions in the kinesin-1 hydrolysis cycle. Proc Natl Acad Sci U S A 112(52):E7186-93.

99. Feuillie C, Formosa-Dague C, Hays LMC, Vervaeck O, Derclaye S, Brennan MP, Foster TJ, Geoghegan JA, Dufrêne YF (2017) Molecular interactions and inhibition of the staphylococcal biofilm-forming protein SdrC. Proc Natl Acad Sci U S A 114(14):3738–3743.

100. Lyubimova A, Itzkovitz S, Philipp Junker J, Peng Fan Z, Wu X, van Oudenaarden A (2013) Single-molecule mRNA detection and counting in mammalian tissue. Nat Protoc 8(9). doi:10.1038/nprot.2013.109.

101. Itzkovitz S, Lyubimova A, Blat IC, Maynard M, van Es J, Lees J, Jacks T, Clevers H, van Oudenaarden A (2011) Single-molecule transcript counting of stem-cell markers in the mouse intestine. Nat Cell Biol 14(1):106–14.

102. Taniguchi Y, Choi PJ, Li G-W, Chen H, Babu M, Hearn J, Emili A, Xie XS (2010) Quantifying E. coli proteome and transcriptome with single-molecule sensitivity in single cells. Science 329(5991):533–8.

103. Albayrak C, Jordi CA, Zechner C, Lin J, Bichsel CA, Khammash M, Tay SX (2016) Digital Quantification of Proteins and mRNA in Single Mammalian Cells. Mol Cell 61:914–924.

104. Lenstra TL, Larson DR (2016) Single-Molecule mRNA Detection in Live Yeast. Curr Protoc Mol Biol 113:14.24.1-14.24.15.

105. Palangat M, Larson DR (2016) Single-gene dual-color reporter cell line to analyze RNA synthesis in vivo. Methods 103:77–85.

106. Yang L, Titlow J, Ennis D, Smith C, Mitchell J, Young FL, Waddell S, Ish-Horowicz D, Davis I (2017) Single molecule fluorescence in situ hybridisation for quantitating post-transcriptional regulation in Drosophila brains. Methods. doi:10.1016/j.ymeth.2017.06.025.

107. Godin AG, Varela JA, Gao Z, Danné N, Dupuis JP, Lounis B, Groc L, Cognet L (2017) Single-nanotube tracking reveals the nanoscale organization of the extracellular space in the live brain. Nat Nanotechnol 12:238–243.

108. Dash A, Simmers MB, Deering TG, Berry DJ, Feaver RE, Hastings NE, Pruett TL, LeCluyse EL, Blackman BR, Wamhoff BR (2013) Hemodynamic flow improves rat hepatocyte morphology, function, and metabolic activity in vitro. Am J Physiol Cell Physiol 304(11):C1053-63.

109. Banaeiyan AA, Theobald J, Paukštyte J, Wölfl S, Adiels CB, Goksör M (2017) Design and fabrication of a scalable liver-lobule-on-a-chip microphysiological platform. Biofabrication 9(1):15014.