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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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