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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.

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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, ~50 nm 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 ~100 nm 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)