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Probing molecular choreography through single-molecule biochemistry

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Abstract

Single-molecule approaches are having a dramatic impact on views of how proteins work. The ability to observe molecular properties at the single-molecule level allows characterization of subpopulations and acquisition of detailed kinetic information that would otherwise be hidden in the averaging over an ensemble of molecules. In this Perspective, we discuss how such approaches have successfully been applied to in vitro-reconstituted systems of increasing complexity.

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Probing molecular choreography through single-molecule biochemistry

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Single-molecule approaches are having a dramatic impact on our view of how proteins work. The ability to observe molecular properties at the single-molecule level allows us to characterize subpopulations and obtain detailed kinetic information that would otherwise be hidden in the averaging over an ensemble of molecules. In this Perspective, we discuss how those approaches are successfully applied to *in vitro* reconstituted systems of increasing complexity.

New fluorescence imaging methods allow us to follow, often in real time, biochemical processes at the single-molecule level and enable the direct observation of conformational dynamics, protein movement and enzymatic activity. Supported by structural and biochemical information, single-molecule approaches allow the design of incisive *in vitro* assays to test mechanistic hypotheses that connect structure to function. Many methodological challenges still exist, including a need for fluorophores with improved photostability and coupling specificity, and better strategies to deal with the analysis and interpretation of highly heterogeneous data. However, the field is rapidly maturing and single-molecule methods are increasingly used as part of an integrated approach with other techniques to achieve mechanistic understanding.

The variety and complexity of the biological systems probed by single-molecule biochemical tools has seen tremendous growth. Originally, the focus was on a relatively limited number of systems that included nucleic-acid based

proteins[1-4] and molecular motors such as kinesin and myosin[5-7], mainly because of the practical accessibility of such systems and a lesser demand on advanced biochemical tools to study them. More recently, other areas in biology have seen enormous benefit from study at the single-molecule level. Further, the application of hybrid detection modalities[8-10], the collection of data on multiple observables[11,12], and the study of many single molecules simultaneously[13-16], has enabled dissection of the interplay between interacting partners in multi-protein systems in complex environments. These new tools are thus providing deeper and wider insight into the molecular mechanisms that underlie many processes that define life, both in their variety and in terms of their complexity. Here, we discuss recent applications of single-molecule approaches, in particular those relying on fluorescence, to the *in vitro* study of proteins in complex environments and systems. In particular, we will use recent work on systems embedded in membranes and studies of large and dynamic multi-protein complexes to illustrate not only the advances in methodology, but also new insights into the dynamic behavior of complex systems.

Complex environments

All molecular processes that support cellular activity take place in complex, crowded environments. Traditionally, biophysical and biochemical studies have focused on characterization of reactions and processes as they occur in the relatively non-complex environment of dilute aqueous solutions. The strength of traditional *in vitro* experiments lies in their capability to control the environment in non-physiological ways, to modify proteins, change concentrations of reagents, and to vary conditions to characterize molecular mechanisms. More recently, biologists have started directly to visualize molecular processes at the single-molecule level in living cells (*in vivo*)[17-19], allowing the study of how molecular processes can be affected by crowding, membrane compartmentalization and other aspects of the intracellular environment. An important goal is understanding the impact of complex environments on biological function. Here we briefly discuss recent single-molecule efforts that

have focused on the characterization of solution-based biochemical processes in crowded conditions and on the study of molecular systems in membranes.

Little systematic effort has been applied to carefully and fully characterize the effects of molecular crowding on biophysical processes. Thermodynamically, excluded volume and ligand depletion effects impact on the free energies of intermolecular interactions[20-22] and altered mobility and anomalous diffusion change the rates at which molecules find and interact with each other[23,24]. Recent single-molecule fluorescence studies have started to highlight the role simple crowding agents such as PEGs can play in stabilizing conformational states[25] and binding lifetimes[26], but there are many more molecular features of the intracellular environment that contribute to crowding. The presence of a large number of differently sized macromolecules creates an environment where the classical notion of molecular diffusion breaks down and percolation effects and anomalous diffusion control the kinetics of reactions[24]. The organization of the cell by cytoskeletal elements and highly structured compacted DNA impose boundary conditions that are yet to be fully recapitulated by experiments. There is a clear need for a deeper physical understanding of how complex environments control salient aspects of molecular properties, such as diffusivity, association rates, motor activity, and others. Given the recent increase in knowledge of molecular mechanisms of many important biomolecular processes and the development of novel biophysical tools that include single-molecule imaging, now is the time to relate our improved understanding of how proteins work to their activities in physiologically relevant environments, to allow us to understand their kinetics and thermodynamics in an environment that more closely mimics the cellular context.

A related direction involves single-molecule studies of membrane-bound systems. Lipid membranes are essential for all cellular life. They compartmentalize metabolic processes, allow for the accumulation of metabolites, and restrict the entry of foreign pathogens and damaging chemicals. A quarter of human genes code for transmembrane proteins[27] and they represent targets for more than half of all drugs[28]. The use of *in vitro* single-molecule fluorescence methods to study membrane proteins has only recently gained traction, in part due to the many technical challenges in production and

handling of membrane proteins, the requirement often for specific labeling and the ability to specifically immobilize the proteins on a surface. The initial focus has been on detergent-solubilized proteins rather than membrane-embedded ones[29-32]. For example, the conformational dynamics of transporter proteins that shuttle small-molecule ligands across membranes were visualized by biotinylating fluorescently labeled and detergent-solubilized protein, coupling it to the surface of a microscope coverslip, and visualizing FRET at the single-molecule level (Figure 1a)[32]. Even though these approaches provided insightful information on protein function, membrane proteins can behave radically differently in detergent micelles than in a lipid membrane[33] and vectorial processes cannot be studied in detergent. It is therefore important to develop more sophisticated methods for the single-molecule characterization of membrane-reconstituted systems.

The glutamate transporter homolog Glt_{Ph}, a homotrimeric transmembrane protein that shuttles aspartate substrate across the membrane[34] has played a key role in the development of new approaches. Substrate transport is mediated by a series of motions of the three transport domains relative to the static trimer scaffold. Crystal structures of the protein in different conformations representing different stages of the transport cycle have been determined, but the order of motions and the level of synchronization among the three subunits was poorly understood[34-37]. Initial single-molecule FRET experiments in a detergent-solubilized system showed the highly dynamic nature of the protein even in the absence of substrate[32]. Reconstitution of Glt_{Ph} in a lipid vesicle (Figure 1b) and visualization of the protein dynamics at the single-molecule level provided insight into the relative motion of the three transporter domains within the trimer[38]. Finally, single-molecule studies on lipid-reconstituted mutant proteins revealed a dynamic transition between locked and free states that display different levels of transport activity[39].

These FRET experiments demonstrate the unique ability of single-molecule approaches to directly visualize dynamics of proteins that otherwise would be hidden in population averaging. The single-molecule way of observing protein motion not only reveals the kinetic properties of transitions among different conformational states that correspond to those observed in structures,

but also highlights the occurrence of rare events and switching between different states of activity. The next logical step in development of such approaches is to combine the observables that report on protein motion to those that show its coupling to ligand transport across the membrane (Figure 1c). The combination of recently developed fluorescent biosensors[40-42] or electrophysiological measurements[43] with single-molecule FRET studies on membrane transporters will provide a quantum leap in understanding of the molecular mechanisms underlying membrane transport and their relevance to disease.

Another class of membrane proteins whose context is crucial to understanding their function are the proteins involved in viral membrane fusion. Membrane-enveloped viruses such as influenza, dengue and HIV contain a lipid-bilayer membrane surrounding internal protein and nucleic-acid components. During entry, the viral membrane fuses with a membrane of the host cell and releases the viral chromosome – a process that requires specific, virally encoded, transmembrane envelope proteins[44]. Recent single-molecule fluorescence imaging with stained viral particles allowed visualization of the kinetics of multiple intermediate steps in the viral fusion process[45-48]. Simultaneous imaging of labeled antibodies that recognize the fusion proteins to inhibit fusion enabled a particle-by-particle analysis of the relation between the number of antibodies bound and the kinetics of fusion[49].

These studies on individual viral particles have greatly contributed to a picture in which successful fusion of the viral and target cell membranes critically depends on the simultaneous action of more than one fusion protein on the surface of the virion. Thus, study of the conformational rearrangements of these proteins will need to be done in the context of intact, native particles. A significant step forward was recently achieved by the visualization of FRET with a single HIV fusion protein on the surface of a native virion[50,51]. The major challenge to overcome is the specific labeling and detection of only a single protein amidst many identical copies. By producing mutant protein with recognition sequences for labeling and by co-expressing them with wild-type protein without tags, viral particles could be produced that had maximally one labeled fusion protein, surrounded by unlabeled ones. These studies highlighted the dynamic nature of protein conformation and the role of ligands in shifting a

conformational equilibrium rather than inducing an all-or-none structural switch.

Complex systems

In addition to visualizing proteins in their physiologically relevant environments, single-molecule approaches can also be extended to multi-protein systems to understand how individual proteins work within larger machines. Such studies will further elucidate how molecular pathways work inside living cells and bridge the gap between *in vitro* studies and observations in a cellular context.

A key challenge is the ability to visualize as many colors as possible to enable the simultaneous detection of distinct molecular species. Such an approach would provide information on stoichiometries of different subunits within a single complex and the order of the events that underlie complex assembly, disassembly, or activity. A great recent example of such an approach is the visualization of the assembly of the protein complex that defines the chromosomal locations where DNA replication starts in eukaryotic organisms[52]. Before DNA is replicated in the S phase of the cell cycle, a large multi-protein assembly called the pre-replication complex (pre-RC) is established in the G1 phase at a large number of chromosomal sites[53]. A carefully orchestrated series of molecular recruitment steps loads precisely two copies of the Mcm2–7 replicative helicase to support the two replisomes needed for bidirectional replication[54]. Helicase recruitment is preceded by the assembly of the pre-RC protein factors ORC, Cdc6 and Cdt1. While the molecular processes underlying assembly of the pre-RC were well studied, the order of events and the precise stoichiometries were unclear. A recent single-molecule fluorescence imaging study relied on the simultaneous use of multiple excitation wavelengths[55] to visualize the relative order of association of the pre-RC factors and to quantify the number of each of the proteins in the complex[52]. Careful analysis of the association kinetics of each of the two Mcm2–7 helicases revealed that the two loading steps rely on different molecular mechanisms. The ability to simultaneously visualize multiple molecular species at the single-molecule level

and observe their interaction kinetics within larger complexes will play an important role in improving our understanding of how large multi-protein machines become established.

Another challenge in applying single-molecule fluorescence approaches to study of large, multi-protein systems is the ability to selectively visualize individual molecules under conditions of high concentration. Single-molecule fluorescence experiments are only possible when the background concentration of fluorescent species is low enough that no more than one fluorescent molecule is present per diffraction-limited detection volume. However, assembly of a complex of various protein constituents requires they be present in concentrations that are sufficiently high to accommodate the affinities of individual protein–protein interactions. While labeling only a small sub-fraction of a particular molecular species permits visualization of association and dissociation kinetics of individual molecules, this approach is not amenable to observation of two different species with different labels. To circumvent this concentration problem, methods have been developed that significantly reduce the sample volume and thus lower the average number of detected background molecules[56,57] or that selectively switch on only the fluorescence of the proteins of interest[58,59]. The latter is achieved either by relying on the longer time that molecules associated with a macromolecular complex reside in the detection volume compared to those that are freely diffusing[58], or by selectively activating the fluorescence of only those molecules that are associated with a macromolecular complex containing a photo-active ligand[59].

An important next step is the development of single-molecule approaches to study interactions *between* macromolecular complexes. Recent work used hydrodynamically stretched DNA molecules to study the outcome of collisions between bacterial replication machines and a DNA-bound replication termination complex[60]. It was known that, only some of the replication forks were stopped at the termination barrier at any particular time in an ensemble of cells. This inefficient fork blockage could reflect either a subset of barriers being bypassed or individual forks being arrested only transiently. To resolve this issue, the authors relied on simultaneous wide-field observation of many mechanically stretched DNA molecules to show forks are permanently stopped,

but only some of the time. By investigating these collision events at the single-molecule level, they also demonstrated a direct relationship between the rate of replication and the probability of termination. As the biochemical yields of properly assembled larger complexes and ability to set up various reaction scenarios diminish with the complexity of the system, it is important to develop multiplexed methodology that allows the acquisition of single-molecule data for hundreds or thousands of individual molecules at the same time. The resulting increase of data throughput is crucial to achieve statistical significance and to conclusively identify rare events and subpopulations.

The ability to characterize subpopulations and parallel pathways as they are reported by single-molecule visualization of complex reactions is having a marked influence on how we think about biochemical processes. The access to subpopulations and rare events offered uniquely by single-molecule studies is showing us that the behavior of dynamic multi-component systems is not as linear and deterministic as ensemble experiments in classical biochemistry often suggest. While the strength of classical ensemble studies is that they enable control of conditions to uncover predominant reaction pathways, it is also has the potential to bias the ultimate interpretation of data in complex systems.

Take bacterial and phage DNA synthesis as examples. The textbook view of simultaneous, coordinated synthesis of the two DNA strands at replication forks is derived from many carefully controlled ensemble experiments. This view is of an ordered and orchestrated series of events where protein components come and go or change binding partners as they function[61,62]. However, there is no chemical reason why this should be so. The DNA replication process has a few irreversible chemical steps like nucleotide incorporation and ATP hydrolysis interspersed by multiple interchanges of protein–protein and –nucleic acid interactions that are often weak and transient, governed by binding equilibria of different energies. In this situation, varying populations of all possible equilibrium states will exist between the irreversible steps, which occur by selection of the appropriate state(s) from these equilibrium populations. This enables ordering of the overall process, but the intermediate thermodynamic states are potentially chaotic. This potential for chaos can, however, be simplified by using a single site on one key protein for interaction with multiple binding

partners, so that binding of two partners at that site is mutually exclusive. Examples of such protein interaction hubs during bacterial DNA replication include the β sliding clamp on double-stranded DNA and the single-stranded (ss) DNA-binding protein (on ssDNA)[63].

A simple example of these principles in action is provided by the *E. coli* replication apparatus, where ensemble experiments show that β clamps are left behind when the associated polymerase is recycled from the end of a completed Okazaki fragment on the lagging strand. In ensemble experiments, clamps need to be provided in excess, and at equilibrium the clamp loader assembly in the replication complex will usually have a clamp bound to it for loading onto an RNA primer for new Okazaki fragment synthesis. Single-molecule experiments, however, were able to show that in the absence of excess clamp, the clamp loader now became available to recycle the clamp[64].

Thus, a dynamic multi-protein system can have many different pathways to achieve a goal, and the way it gets there will be largely controlled by the kinetic and thermodynamic boundary conditions it encounters along the way. A multi-step process involving many partner proteins and transient interactions among them can be seen as a path taken through a complicated energy landscape: many routes can lead to the same end state. In a way, biochemical processes that involve many steps and partners will likely turn out to be messier than we thought. This viewpoint represents a shift in how we think about complex biochemical problems and underscores the notion that our understanding of the world around us is only as good as our ability to see it.

Figure captions

Figure 1. (**a**) In earlier methodology, individual detergent-solubilized proteins (tan rectangles) were immobilized by direct coupling of the biotinylated protein to streptavidin (green) bound to a microscope cover slip (gray platform). The protein was labeled with donor and acceptor fluorophores (red and yellow spheres) to facilitate the observation of conformational dynamics. (**b**) More recently, single-molecule FRET experiments have been performed on membrane transporters reconstituted into a lipid vesicle (blue ring), thus allowing for the application of concentration gradients. (**c**) An important next step is the simultaneous observation of conformational dynamics of individual transporters or channels through FRET while monitoring ligand transport by means of fluorescently labeled biosensors (orange sphere) or electrophysiological tools (gray dial). Substrate molecules are indicated as black triangles; biotin is indicated as black circles.

Figure 2. In contrast to ensemble-averaging bulk methods, single-molecule methods provide direct information on stoichiometry, order of assembly, conformational dynamics, subunit exchange and interactions with other complexes.

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Erkens *et al*., Nature (2013)
By reconstituting FRET-labeled transporter proteins in vesicles, the authors visualized conformational changes associated with transport in the context of a lipid membrane.

Munro *et al*., Science (2014)
The authors labeled a small fraction of fusion proteins on the surface of HIV particles with FRET probes and visualized conformational changes of the individual fusion trimers.

Ticau *et al*., Cell (2015)
By fluorescently labeling the different protein factors involved in eukaryotic replication licensing, the authors visualized protein stoichiometries and orders of assembly on the DNA.

Elshenawy *et al*., Nature (2015)
By visualizing at the single-molecule the collisions between the replication machinery and the replication-termination complex of *E. coli*, the authors showed that only replisome traveling at a certain rate are halted.

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Figure 1

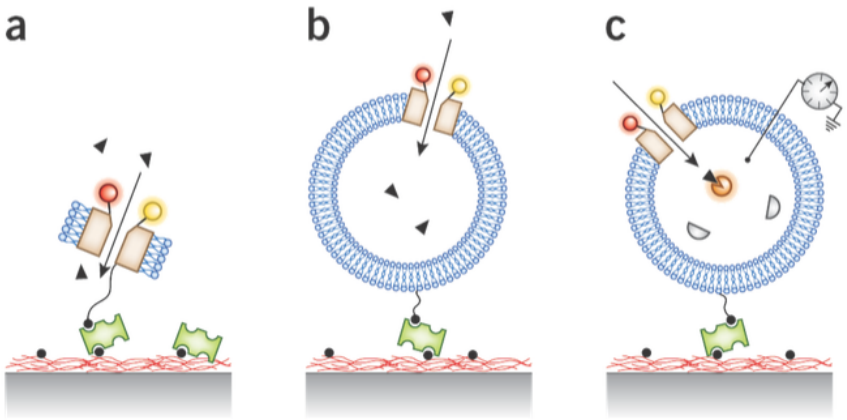

Figure 2