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Review

In vivo single-molecule imaging of bacterial DNA replication, transcription, and repair

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ABSTRACT

In vivo single-molecule experiments offer new perspectives on the behaviour of DNA binding proteins, from the molecular level to the length scale of whole bacterial cells. With technological advances in instrumentation and data analysis, fluorescence microscopy can detect single molecules in live cells, opening the doors to directly follow individual proteins binding to DNA in real time. In this review, we describe key technical considerations for implementing in vivo single-molecule fluorescence microscopy. We discuss how single-molecule tracking and quantitative super-resolution microscopy can be adapted to extract DNA binding kinetics, spatial distributions, and copy numbers of proteins, as well as stoichiometries of protein complexes. We highlight experiments which have exploited these techniques to answer important questions in the field of bacterial gene regulation and transcription, as well as chromosome replication, organisation and repair. Together, these studies demonstrate how single-molecule imaging is transforming our understanding of DNA-binding proteins in cells.

1. Introduction

Protein–DNA interactions are critical to many important biological functions, from gene regulation and transcription to DNA replication and repair. To better understand these processes we need to look at molecular details, such as the intracellular concentrations and stoichiometries of DNA-binding proteins, the location of DNA binding sites inside cells, and the binding kinetics of these proteins. However, focusing on the molecular level can miss the bigger picture; we also need to understand how protein–DNA interactions shape the organisation of chromosomes and cause phonotypical changes over the whole cell.

In vitro single-molecule experiments have extended our understanding of protein–DNA interactions based on detailed mechanistic analysis using purified proteins and DNA oligonucleotides. On the other hand, proteins can be imaged inside living cells with fluorescence microscopy. However, while conventional fluorescence microscopy can report on large cellular features, details are lost below the diffraction limit of light (~250 nm). In vivo single-molecule imaging bridges the gap between molecular-level and cellular-level experiments, and can thus address new questions that neither in vitro studies nor conventional fluorescence microscopy can answer.

Knowing where proteins bind DNA is central to understanding their function. Tools like chromatin immunoprecipitation (ChIP) are powerful in determining specific binding sequences [1], but they give no information on where these binding events take place spatially within cells, and what their kinetics are. Because these assays use lysates from many cells, they can only report on population averages. In vivo single-molecule techniques can allow individual DNA binding events to be observed in live cells [2–10], shedding new light on binding behaviour, and resolving heterogeneity that ensemble measurements can miss [11,12]. Moreover, these techniques can report on the spatial locations of these actions in cells, and how they change as cells respond to stimuli [4]. Experiments in live cells can also report on the kinetics of processes. For example, measuring protein dwell times on DNA in live cells provides insight into the rates of enzymatic action of proteins [4]. The rates of DNA binding can also shed light on the mechanisms by which proteins locate their specific binding sites [2,3].

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In this review we give an overview of the techniques involved in imaging single molecules in live bacteria and show how these can be combined with tools like single-particle tracking to offer a new perspective on protein–DNA interactions from the molecular length scale to the level of whole bacterial cells. We highlight experiments that used these methods to solve key questions in bacterial transcription, DNA replication, and repair.

2. Single-molecule methods

2.1. In vivo single-molecule fluorescence

Fluorescence microscopy allows labelled molecules to be observed inside cells with greatly reduced unwanted signal from other cellular components. This specificity naturally lends itself to single-molecule studies; however, to image individual fluorophores in vivo several experimental challenges need to be overcome.

Single-molecule fluorescence microscopes aim to maximise the signal collected from each fluorophore by using high numerical aperture objectives together with sensitive cameras. Laser excitation reduces unwanted background fluorescence due to the narrow frequency spectrum. Sources of fluorescent contamination can be minimised, for example, by preparing cells with low fluorescence growth media, and carefully cleaning cover slips to remove contaminants [13]. Total internal reflection fluorescence (TIRF) microscopes are popular for single-molecule imaging as they reduce the problem of out-of-focus fluorescence, which is inevitable in epifluorescence systems. In TIRF illumination, the excitation beam is reflected at the interface between the coverslip and the imaging medium so that the resulting evanescent excitation extends only ~150 nm into the sample [14]. This technique is well suited to studies focused on the cell membrane, but less appropriate for imaging deeper into the cytoplasm. To image the bacterial nucleoid, TIRF systems can be used at sub-critical angles giving a thin sheet of excitation light at a shallow angle to coverslip [15].

The fluorophore used to label the protein of interest is of crucial importance to the sensitivity of the experiment. Synthetic dyes are generally brighter and more photostable than fluorescent proteins, however they also have major disadvantages. Immunostaining is a common tool to label with synthetic dyes, but it requires cells to be chemically fixed and permeabilised. Unknown labelling stoichiometries of fluorophores per antibody and incomplete staining of a particular protein with antibodies in the cell can hinder quantitative analysis of the microscopy images [16]. Furthermore, delivering synthetic dyes into live cells is problematic, although progress is being made, as discussed in Section 6 [17–21]. In this review, we focus on labelling with genetically encoded fluorescent proteins. While not as bright or photostable as synthetic dyes, fluorescent proteins are ideal for imaging live cells and can achieve almost 1:1 labelling stoichiometry which facilitates counting proteins.

2.2. Super-resolution microscopy

Several microscopy methods have been developed to resolve molecular structures smaller than the diffraction limit of light (~250 nm). Stimulated emission depletion microscopy (STED) [22] increases resolution by supressing unwanted fluorescence surrounding the central excited area, achieving a resolution of 50–70 nm for fluorescent proteins [23]. In structured illumination microscopy (SIM) [24,25] multiple images are taken with patterned excitation light to extract additional spatial information about the sample, allowing a resolution of ~100 nm [23]. Both these techniques, however, image ensembles of molecules [23].

The microscopy image produced by a single fluorophore is a finite-sized spot whose size is limited by diffraction. The shape of this intensity profile is known as the point spread function (PSF) and can, in many cases, be well approximated by a Gaussian function [26]. This allows the exact position of an isolated molecule to be determined with much higher precision than the size of the PSF by fitting the image to a Gaussian mask (Fig. 1A I) [27]. The uncertainty of the fitted position depends mainly on the number of photons collected [26,28]; for typical single-molecule experiments using fluorescent proteins, the uncertainty is between 10 and 50 nm.

In order to fit the fluorescence intensity profile of a molecule, it must be sufficiently separated from any other molecules to be clearly spatially resolved. Due to the small size of bacteria, this means imaging only a few molecules per cell; a much lower density than most proteins in *Escherichia coli* (Fig. 1A II). Photoactivated localisation microscopy (PALM) overcomes this problem [29] by taking advantage of photoconvertible or photoactivatable fluorescent proteins, such as mEos2 [30], Dendra2 [31] or PAmCherry [32]. These proteins can be photoactivated with UV light, the intensity of which can be chosen to ensure that there are very few emitting (photoactivated) molecules at any given time. Molecules are stochastically activated, imaged and localised over a movie with typically several thousand frames. The localisations from all frames can then be reconstructed into a super-resolved image [23,29,33,34].

2.3. Single-particle tracking

Tracking the movement of molecules in live cells is a powerful approach that in principle allows directly observing the kinetics and location of protein activities. Single-particle tracking (SPT) algorithms join together the positions of molecules over a series of images to form trajectories. The density of labelled molecules should be low so that their PSFs are resolved and trajectories do not cross. This requirement limited early studies in bacterial cells to artificially low protein copy numbers, as illustrated in Fig. 1A II [2].

Combining single-particle tracking with the strategy of photoactivation central to PALM allows many molecules to be tracked sequentially [35], making it possible to study a much broader range of biological problems. The basis of this technique is illustrated in Fig. 1B. As in typical PALM studies, single molecules are sparsely photoactivated and imaged for a number of frames until they photobleach. Compared to ordinary PALM, lower excitation intensities are used to allow molecules to be tracked for a longer duration at the cost of decreased localisation precision.

2.4. Analysing protein diffusion

Studying the mobility of DNA-binding proteins can provide key insights into their function. Several well-established techniques exist for obtaining information about the diffusive motion of molecules inside cells, such as fluorescence correlation spectroscopy (FCS) [36] and fluorescence recovery after photobleaching (FRAP) [37]. In FCS the mobility of molecules is inferred from the timescale of fluorescence intensity fluctuations as they diffuse through a focussed excitation beam. FRAP measures the time it takes for fluorescent molecules to diffuse into a previously photobleached area. Fitting FCS and FRAP data to the appropriate model can allow information on binding kinetics, such as association and dissociation rates, to be extracted [38].

While both these techniques can be used to great effect, they are limited by the fact that ensemble measurements can mask the presence of multiple molecular species with different diffusion behaviour through averaging [39]. As a consequence, care must be taken when extracting binding kinetics based on a pre-conceived kinetic model [38]. Both FRAP and FCS study diffusion within a single spot in the cell at any given time. In contrast, single-particle