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Single molecule techniques in DNA repair: A primer

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ABSTRACT

A powerful new approach has become much more widespread and offers insights into aspects of DNA repair unattainable with billions of molecules. Single molecule techniques can be used to image, manipulate or characterize the action of a single repair protein on a single strand of DNA. This allows search mechanisms to be probed, and the effects of force to be understood. These physical aspects can dominate a biochemical reaction, where at the ensemble level their nuances are obscured. In this paper we discuss some of the many technical advances that permit study at the single molecule level. We focus on DNA repair to which these techniques are actively being applied. DNA repair is also a process that encompasses so much of what single molecule studies benefit – searching for targets, complex formation, sequential biochemical reactions and substrate hand-off to name just a few. We discuss how single molecule biophysics is poised to transform our understanding of biological systems, in particular DNA repair.

1. Introduction

1.1. The development of single molecule techniques

Studying systems at the single molecule level is becoming more widespread, however the field is still relatively new, finding its origins in the Nobel prize winning work on single ion channels [1]. These channel studies provided not only the concept that single molecules could be investigated, but also a huge number of tools; theoretical, practical and analytical. A key concept from this work is that ensemble level studies provide an overview of the average behavior of a molecular species. This seemingly obvious statement belies its importance, because during a biological process one or more proteins explore their energetic landscapes potentially through a multiplicity of pathways. The ensemble is the sum of all of these processes, however, a single molecule recording deconstructs this average; providing the discrete probability of passing through each constituent pathway. The importance of understanding molecular systems, at this level, is evident as one attempts to scale up toward more complex systems [2]. In the context of DNA repair, understanding systems one molecule at a time permits a view of the heterogeneity of the molecular states involved in a biochemical reaction. On the nanoscale both protein structures DNA structures fluctuate, it is only when these structural fluctuations coincide in the correct manner that a chemical reaction occurs. A structural snapshot of a process provides one final or intermediate state, but not the multiplicity of states, their significance, or their dynamics. Ensemble approaches to obtain dynamics require aligning the biochemistry of all of the molecules, for example during a 'single turnover' experiment [3]. This provides information on the behavior of proteins for a limited time, since they become asynchronous very quickly. In the steady state, each interaction at the single molecule level is a single turnover and therefore does not require synchronization. Furthermore, many repair reactions require multiple protein partners that assemble at the damaged site; therefore through single molecule analysis it is possible to follow the fate of a particular reaction from initial DNA damage location to completed repair. The ultimate goal of single molecule studies is the analysis of a complete reaction mechanism with dynamical information on the comings and goings of protein components. In other words, watching a full biochemical reaction unfold in real time.

1.2. Limitations of single molecule experiments

Although single molecule approaches are extremely useful there are crucial limitations. The main barrier to studying any system at

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the single molecule level is signal. Even in ensemble experiments with 1 mL of 1 mM solution, signal can be weak, despite the amplification offered by 10^{17} molecules. For single molecule experiments, no such inherent amplification is present. Instead amplification must come from external sources such as detectors. Therefore, dealing with such low signals above noise has required the development of new technologies. These utilize electron-multiplying CCD (charge coupled device) imaging cameras with unprecedented sensitivity and more recently large-sensor CMOS (complementary metal oxide semiconductor) cameras that possess incredibly fast frame rates [4]. From the aspect of fluorescence imaging, however, it is irrelevant how fast a camera records if the photons are not sufficient to generate a signal. Therefore new fluorophores have been derived that are extremely bright [5]; these however, in turn need to be balanced against photostability. This latter problem occurs when a fluorophore undergoes a covalent modification during the population of an excited state, resulting in loss of the ability to emit photons [6]. To offset this problem a number of groups have derived fluorophores with various emission wavelengths possessing enhanced quantum yields and photostability [7]. Also a broader understanding of fluorescence photophysics has generated solution additives that delay photobleaching [8]. One huge advance has been the development of quantum dots (Qdots) for biological applications [9–12]. These semi-conductor based fluorophores possess several unique properties: they are brighter and much more photostable than their organic counterparts and they emit over a more narrow wavelength range. In addition, their unique broad excitation spectra allow multiple Qdots to be excited by a single wavelength source; thus permitting true simultaneous multi-color imaging.

This review discusses how single molecule biophysics has been used to study the process of DNA repair. As a prelude to the articles within this special issue of *DNA Repair*, we aim to highlight the ingenious methods used to examine systems at this level focusing on imaging, manipulation and then how these may be used to study systems within cells.

2. Imaging based methods – a bright future

Real time imaging of protein–DNA interactions can provide information both on the compositional and dynamic aspects of repair. There exist a vast range of single molecule studies performed with DNA binding proteins, which have provided information on the search mechanism, as well as, the diffusion constants of the protein/complexes [13]. A few examples of the imaging techniques used to study different DNA repair systems and the data acquired using them are outlined here.

2.1. Tethered particle motion

First used over two decades ago [14,15] tethered particle motion (TPM) detects the position of a bead attached to DNA at one end when the other end of the DNA is attached to a surface. The surface tethering of the DNA restricts the inherent Brownian motion of the bead in aqueous solution (Fig. 1). This restriction is proportional to the length of the tether [14], therefore the amount of Brownian motion provides a signal for DNA shortening events. This method is well suited to studying proteins that bend, loop or translocate along and hence shorten the DNA [16]. TPM has many advantages such as being simple and inexpensive with temporal resolution high enough to detect the rapid kinetic behavior of individual molecules. No external force such as flow is required during this type of investigation and TPM can be combined with other methods, such as optical trapping (see below). TPM has been used to monitor the processivity of RecBCD helicase, which is responsible for initiating double-strand break DNA repair through homologous recombination [17]. This investigation demonstrated that RecBCD can translocate along DNA, and its interaction with a regulatory 8 nt Chi sequence (5′-GCTGGTGG-3′) did not alter its translocation velocity. However, subsequent alterations to TPM using stretching forces, induced by the introduction of a continuous buffer flow into the sample chamber, increased positional accuracy, such that reduced translocation velocity could be detected in the Chi regions [18].

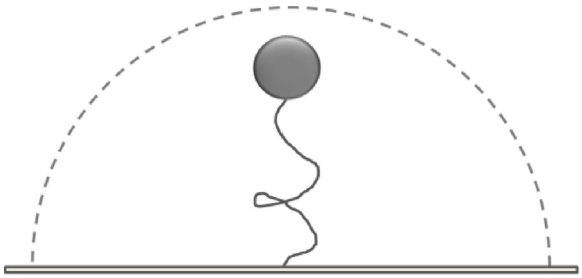

Fig. 1. The tethered particle movement assay. A single DNA molecule is tethered between the slide surface and a bead particle. The dash line represents the range of Brownian diffusion of the microsphere, which depends on the DNA contour length.

TPM has also been used to study RuvA, a protein involved in mediating branch migration and Holliday junctions created during homologous recombination in bacteria. Again a slight alteration to TPM was used where, instead of averaging an image over time, the individual trajectories of the beads were measured [19]. This allowed bead positioning with nanometer-scale precision. The study was able to view in real time RuvA-mediated unfolding of the Holliday junctions, detected as an increase in the length of the DNA tether.

2.2. Combing

DNA normally forms a collapsed bundle in solution with a diameter that can be approximated by random flight theory (for λ-DNA this is ~6 μm [20]). This makes imaging protein attachments to DNA particularly challenging, therefore investigators typically elongate or extend the DNA into a linear chain. One way this is performed is through the DNA combing technique. DNA is laid directly onto the surface of a cover slide in a combing assay. Firstly, the surface of the slide is activated with a hydrophobic moiety such as polystyrene or polymethylmethacrylate [21]. Then, either using continuous flow [22] or retracting a slide through an air–water interface [23]. DNA can be stretched between two or more points in low pH conditions. Imaging is performed using Total internal reflectance fluorescence (TIRF) microscopy, where the evanescent field provides high signal to noise [21,22]. Combing has the advantage of being technically simple, however, there are a few disadvantages; the DNA and the proteins are both imaged on the surface of the slide, meaning it can be difficult to distinguish fluorophores on the surface from those on the DNA. In addition, the surface itself may have profound effects on the protein–DNA interaction and/or the native states of both.

For DNA molecules that are surface bound from only one end, continuous flow can be applied to extend the DNA [24,25], overcoming some of limitations of pure combing. This approach has been used to study the base excision repair protein human oxoguanine DNA glycosylase1 (hOGG1) in a landmark study for single molecule imaging of DNA repair [24]. This study attempted to settle the facilitated diffusion paradox. By directly imaging the motion of hOGG1 on DNA it was possible to derive the diffusion constant of a molecule on DNA. From this value rotation-coupled diffusion was inferred and the energetics of the protein–DNA interaction could be

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