Single molecule transcription elongation

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ARTICLE INFO

ABSTRACT

Single molecule optical trapping assays have now been applied to a great number of macromolecular systems including DNA, RNA, cargo motors, restriction enzymes, DNA helicases, chromosome remodelers, DNA polymerases and both viral and bacterial RNA polymerases. The advantages of the technique are the ability to observe dynamic, unsynchronized molecular processes, to determine the distributions of experimental quantities and to apply force to the system while monitoring the response over time. Here, we describe the application of these powerful techniques to study the dynamics of transcription elongation by RNA polymerase II from Saccharomyces cerevisiae.

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1. Introduction

The techniques for studying single molecule eukaryotic transcription are based on the innovation of dual trap optical tweezer assays [1–4], the work of those who have advanced the in vitro study of RNA polymerase II (RNAP II) elongation [5–8] and the development of similar assays for the study of Escherichia coli RNA polymerase [9–12]. The experiments are designed to record the positions of an individual polymerase along the DNA template as a function of time. There are three advantages of collecting data from a single enzyme in the context of an optical tweezer setup: (1) the ability to observe dynamic events that would be unsynchronized if studied in bulk, (2) the measurement of distributions of observables rather than just their mean and (3) the application of a perturbing force to the system.

It has been known for more than 25 years that cellular RNA polymerases do not transcribe their templates with uniform velocities [13]. Instead, transcription elongation is interrupted by a variety of pause states [14] that play roles in many aspects of transcription regulation including promoter proximal escape [15,16], termination [17,18], nascent RNA folding [19], polyadenylation [20] and splice site selection [21]. These pause states have been studied extensively by a variety of techniques including gel electrophoresis, stop flow spectroscopy and other methods that derive the average behavior from a population of molecules. The proper analysis of these methods requires that the population be synchronized in such a way that all molecules occupy the same nucleotide position at the beginning of the experiment. What is missed by these experimental techniques is that pauses occur all over the template in a sequence biased, but stochastic manner. While strong sequence dependent pauses are the most easily studied, their behavior only represents the long time tail of the distribution of all pause times and they account for only a small fraction of the total number of pauses. Furthermore, it is clear that the cellular regulation of eukaryotic transcription elongation is not coded directly in the template sequence, but instead depends on many factors that cooperate (or compete) to determine whether or not a gene is to be transcribed. For example, weak pauses may be strengthened by the presence of factors such as nucleosomes [22] and strong pauses may be bypassed through the binding of elongation factors such as TFIIS [23]. Therefore, understanding pause dependent transcription regulation mechanisms depends on the understanding of general pause mechanisms and their inter-relatedness and requires the observation of all pauses that might occur along a template. Single molecule transcription techniques make it possible to characterize the overall distribution of pause behavior to supplement what is known about specific pause mechanisms that operate within specific sequence contexts.

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Another advantage afforded by the optical tweezers assay is the ability to apply a perturbing external force and to observe the polymerase's response. Far from being a curious detail of interest only to those studying the detailed physics of molecular motors, the performance of work and the generation of force by the polymerase are crucial for both transcription processivity and regulation within the cell. Transcription takes place in a crowded nuclear environment with many physical barriers caused by DNA template bound proteins including histones, DNA repair enzymes, DNA polymerases, other RNA polymerases, topoisomerases, and other proteins involved in DNA metabolism or transcription regulation. Furthermore, due to intervening sequences, genes in eukaryotes can stretch up to millions of base pairs requiring RNAP II to make its way through a considerable amount of traffic [24,25]. Therefore, to successfully transcribe a single complete gene, the polymerase must not only bind strongly to the DNA template, but must possess robust mechanisms to perform physical work in the face of barriers and opposing forces.

In this review of methods for the single molecule analysis of RNAP II transcription elongation we review the physics of optical trapping, describe the specific experimental protocols used to acquire and analyze single molecule data, and conclude with a short summary of the important results that have been obtained using these methods.

2. Optical trapping methods

The development of single molecule optical tweezer methods was initiated by the observation that the interaction between light in a highly focused laser beam and micron sized dielectric particles results in the formation of a three-dimensional trap with restoring forces on the order of piconewtons [1]. Here we only review the basics as full descriptions of the principles and practice of optical tweezers have been published elsewhere [26,27]. A ray optics description of light may be used to form an intuition of the interactions between the light and the particle that lead to the formation of the trap [1] (Fig. 1a and b). This description is not physically accurate unless the size of the bead is much larger than the wavelength of the light, however it serves to gain insight into the interaction between the light and the bead. In most biological applications, optical traps are used exactly in the regime where the bead size is on the order of the wavelength where these physical descriptions break down. See [27] for a detailed review of theoretical approaches to this problem.

If the index of refraction of the bead is greater than the index of refraction of the buffer, the light will be bent in the direction of the bead displacement (from the center of the trap) as it passes through the bead. Light carries momentum and thus a change in its direction results in a change of momentum. Since momentum is a conserved quantity, the bead must experience a change in momentum equal and opposite to that experienced by the light. This effect produces a force that acts to restore the bead the center of the beam (Fig. 1a). To create a three-dimensional trap a high numerical aperture lens (i.e. N.A. 1.2) is used to generate high angle rays beams that impinge on the bead (Fig. 1b). By extending the analysis of the refraction of a single ray, we see that in this arrangement, no matter which way the bead is displaced the sum of the effects from the high angle rays will result in a force pulling the bead towards the center of the trap. In practice, trap strength depends on laser power, bead size and other environmental factors and must be calibrated by comparing the strength of the trap to the thermal energy. This procedure is described in more detail in the protocol section below as it is performed on each individual bead pair before each experiment. For distances less than the diameter of the particle, the trap acts simply as a Hookean spring ($F = kx$) where F is the force, k is the trap stiffness and x is the displacement of the bead from the center of the trap. Thus, after the measurement of trap stiffness, the force on the bead may be simply calculated from its position. During an experiment both the force and the position of the individual beads relative to the trap centers are monitored at rates up to 10 kHz and in dual trap setups, the final experimental estimate of the force is obtained through an average of the force on each bead. Typically these forces are very similar and these two independent measurements of the force increase accuracy. The position measurement of the polymerase in a passive mode experiment requires that the bead–trap distances be transformed into bead–bead distances and converted into DNA contour lengths. This is done in practice using the worm-like-chain theory of DNA elasticity that describes DNA as a nonlinear spring and relates its equilibrium end-to-end length to its contour length as a function of force [28,29].

To construct a double trap tweezer, one requires a minimal set of components well aligned on an optical table in a room that minimizes environmental noise and temperature fluctuations (better than OSHA NC30 and ±0.5 °C [27]). Double trap apparatuses have the advantage over single trap tweezers in that they isolate the system from the lab frame and thus possess less noise and drift. This improvement can be crucial for the measurement of long trajectories where enzyme velocities are not very high and on the order of 1–10 nt/s. Furthermore, since data may be acquired from each trap independently, correlations between the beads may be used to reduce noise even further. Lastly, by splitting the laser into two orthogonally polarized beams, fluctuations in the direction of beam propagation that stem from the laser source itself are experienced simultaneously in both traps and do not result in relative motion of the two trapped beads [30]. Fig. 1c shows the layout of the most important features of the setup and more details may be found elsewhere [26]. Briefly, a single 5 W, 1064 nm laser (Spectra-Physics, J20-BL10-106C) is expanded with a telescope (~4×) and then split with a polarizing beam splitter (PBS) into two separately controllable beams that each creates a single trap. A motorized $\lambda/2$-wave-plate positioned before the PBS can be used to control (and equalize) the power of each beam and thus the stiffness of each trap. A mirror mounted on a two axis piezoelectric scanner (Mad City Labs, Nano-MTA series) steers one beam (red) while an acousto-optical device (IntraAction, DTD series) may be used to steer the other beam (orange). The method of steering chosen depends on the specific needs of the instrument and can also be realized with electro-optical devices [31] and moveable lenses. After the steering components, the two beams are recombined with a second PBS, passed through another expanding telescope (~4×) and directed through the objective lens. To create stable traps in this configuration it is necessary to expand the beam sufficiently to overfill the back plane of the objective lens to generate the highest angle rays possible to generate the force along the beam axis as described above (Fig. 1b). The flow cell (Fig. 1d) is positioned between the matched objective and condenser lenses (Nikon Plan Apo VC water immersion objectives, N.A. 1.2) and is controlled through the computer with a three axis stage (Newport, 562 series), motion controller (Newport ESP300) and motorized actuator screws (Newport, CMA series). Lastly, the light from the traps is collected, separated with a third PBS and imaged onto position sensitive detectors (Pacific Silicon, DL100). As the light influences the trapped bead, the position of the bead influences the interference pattern produced between the scattered and unscattered light leaving the trap. Using a technique known as back focal plane interferometry, the movements of the bead may be measured [32,33]. A separate optical path using a light emitting diode (LED) light source and a CCD camera (Watec, 902H2 supreme) is coupled into the objectives and used to image the beads during the experiment and may also be used for automated and independent video tracking of bead positions (Fig. 1e).