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Review

Single molecule approaches to transcription factor kinetics in living cells

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ABSTRACT

Quantitative modeling of intracellular processes often requires information about intracellular rate constants as well as the concentrations of low abundance species in individual cells. Single molecule imaging techniques offer not only new ways for obtaining such information but also the possibilities to test model-based hypotheses that have previously been out of reach for experiments. In this review we highlight some advantages of single molecule techniques and exemplify by their capability to help understanding how transcription factors find their chromosomal binding sites in bacterial cells.

1. Why single molecule methods

Single cell investigations have shown that even an isogenic population of cells in an uniform environment can have very diverse proteome composition [10] and display different phenotypes [5,7,33]. The variation could originate from several sources, including epigenetic phenomena that lock a cell in one of several possible cellular states, copy number fluctuations of molecules due to the stochastic nature of chemical reactions, or sustained oscillations that are out of phase over the population. Whatever the reason for heterogeneity, it is clear that averaging over a population of cells leads to loss of information that is relevant for many systems and their dynamics.

The problems and consequences of averaging in systems biology do not however end with the study of single cells. Even within a single cell, averaging over a population of the same type of molecules may lead to loss of important functional information. For instance, enzymes can be at different states of their catalytic cycle, and proteins can be at different levels of co-factor binding. Even if the different states have their well defined and detectable output, such as fluorescence, diffraction or scattering, transitions between states cannot be monitored as an average over many molecules unless they are synchronized. Single molecule techniques are very powerful in this regard, and have made it possible to elucidate the working of many enzyme complexes in vitro (motors [12], channels [31], ribozymes [42], enzymes [38], polymerases [16], etc.). However, the most powerful applications of single molecule techniques are likely to be in living cells, where it is impossible in principle to synchronize reactions between molecules. It is also often the case in vivo that the copy number of molecules is so low that only a single molecule prospective is meaningful.

As an example of how single molecule techniques can be used to understand biological processes in living cells and to test quantitative hypotheses, we will discuss recent experimental and theoretical advances in the study of how the transcription factor (TF) LacI locates its binding site in the *Escherichia coli* chromosome.

2. Measuring transcription factor search kinetics

Our approach to study how fast TFs locate their targets on the chromosome is based on single molecule fluorescence microscopy [8]. With the state-of-the-art detection system [18], a single TF–YFP fusion protein, when binding to the chromosome, can be imaged as a diffraction-limited spot because of the localized fluorescence [37]. On the other hand, other fusion TFs that are freely diffusing in the cytoplasm result in diffuse fluorescence that is indistinguishable from the auto-fluorescence background. To avoid the overwhelming signal of diffuse fluorescence on top of the signal from a single localized YFP, the expression level of the fusion protein has to be kept low (<10 molecules per cell). The exposure time is chosen such that in this time interval the chromosome movement is insignificant and the freely diffusing TFs have

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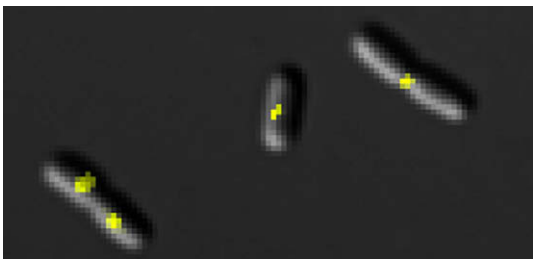

Fig. 1. YFP-labeled transcription factor MalI bound to its chromosomal locus (Uppsala 2008).

explored the entire cell. We are therefore able to observe YFP-labeled TFs when they are specifically bound to DNA (Fig. 1), but not when freely diffusing. This technique makes it possible to study TF binding and dissociation kinetics at a time resolution of seconds, which is approximately two orders of magnitude faster than that of other single cell assays for gene regulation. Since the observable is the localized fluorescence, the method is not limited by the maturation time of fluorescent proteins. Instead, the time resolution is limited by how fast a non-specifically bound molecule can diffuse throughout the bacterial cell (~100 ms).

We employed this technique to measure how long time it takes for a single protein to search for a specific target in the chromosome (Fig. 2). An *E. coli* cell strain is engineered with YFP fused to the *lac* repressor (LacI) expressed at its chromosomal locus. To measure the search time, cells under the microscope are pre-incubated with the inducer (IPTG) so that all LacI molecules have dissociated from their specific binding sites (LacO operators), resulting in loss of localized fluorescence. To initiate the search process of LacI–YFP, we rapidly wash away the inducer and add a high concentration of anti-inducer. Immediately after the medium exchange, the fraction of the TF-bound operators is measured at different time points. The localized fluorescence from single TFs reappears after 1 min. Because the maturation time of YFP (Venus) is 7 min [39], the fluorescence reappearance is not from newly synthesized fusion TFs [36], but instead from the rebinding from the old TFs that have diffused throughout the cytoplasm. The search time for one TF to find one binding site is therefore $\leqslant$6 min, because there are three repressors searching for two binding sites within a diffraction-limited spot in a cell. Is this a reasonable search time for a TF looking through millions of nonspecific DNA sequences? In the following, we use a quantitative model to answer this question.

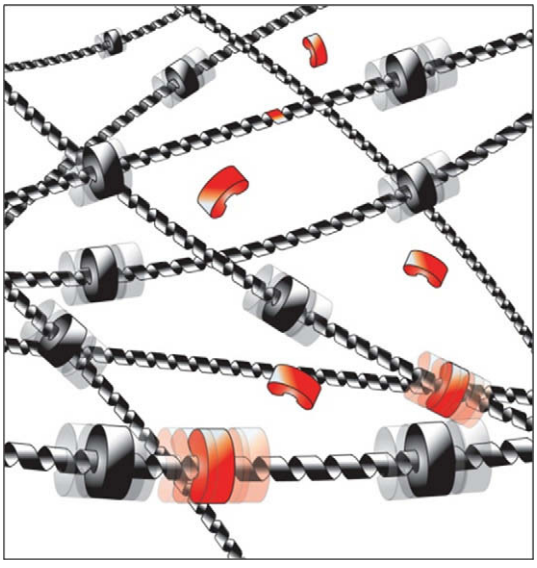

Fig. 2. Facilitated diffusion. The red protein is searching for the target (red DNA segment) using 1D-sliding on the crowded DNA and 3D-diffusion in the cytoplasm [23].

3. Target location by facilitated diffusion

Binding kinetics in vivo is often at the diffusion-limited regime, because intracellular diffusion, both translational and rotational, is significantly slowed down by macromolecular crowding [40]. In this regime, further evolution of an enzyme's intrinsic catalytic rate is no longer advantageous because the processes of finding the substrate become rate-limiting. Many proteins have instead evolved strategies to find their intracellular targets faster than what is allowed by the apparent diffusion limit set by the Smoluchowski expression $4\pi\rho D_3$, for two reactants with reaction radius ρ and translational 3D-diffusion constant D_3 [6].

In particular, DNA-binding proteins such as RNA polymerases, DNA-repair enzymes, restriction enzymes, and transcription factors are believed to combine 3D-diffusion in the cytoplasm with 1D-diffusion (sliding) on nonspecific DNA to find specific binding sites (Fig. 2). This phenomenon is termed facilitated diffusion (as reviewed by von Hippel and Berg [35]). Since 1D-diffusion is very redundant, nonspecific association near the actual binding site leads to target binding with a high probability. Such an antenna effect increases the reaction radius from that of the specific site to the distance that a TF slides along DNA before dissociation. Therefore, for a sliding distance of 30 base pairs (~90 Å), the search speed is increased by a factor of 30 by 1D-diffusion. Similarly, enzymes that operate on membranes can use the surrounding membrane as a 2D-antenna to increase the capture radius and the rate of substrate binding [1].

Besides 1D-sliding, another mode of facilitated diffusion has been proposed. Intersegment transfer is a process in which the TFs can jump between DNA segments. Many DNA-binding proteins have two distant binding domains, which allow the protein to attach to two independent DNA segments and rapidly move from one to the other. However, because the LacI–YFP fusion protein used by us does not tetramerize during the search process, we do not consider the contribution of intersegment transfer here.

4. What is the search time predicted by the facilitated diffusion model?

Many, principally similar, quantitative approaches have been taken to model facilitated diffusion [3,9,15,17,20,26,29,32,41] and here we describe the Smoluchowski-type method.. The bi-molecular association rate constant between the protein and the specific site is [2,23,26]

$$k_a = \frac{2\pi}{\ln(\xi/2b)} DL = \frac{2\pi}{\ln(\xi/2b)} \left(\frac{D_3}{1 + K_{RD} c_{ns}} \right) (l + 2\sqrt{D_1 t_R}) \qquad (1)$$

The first equality describes the rate constant as a product of an effective diffusion constant (D) and an effective target size (L). For the specific model of facilitated diffusion, D depends on the cytoplasmic 3D-diffusion constant (D_3), the base pair concentration of nonspecific DNA (c_{ns}), and the nonspecific binding constant (K_{RD}). L depends on the diffusion (sliding) rate constant on DNA (D_1), and the residence time (t_R) on non-specific DNA. The logarithmic factor corrects for short-ranged microscopic dissociation/association events (hopping) along the same DNA segment [3]. The ratio $\xi/2b$ compares the length scales of macroscopic verses microscopic

dissociation. In the current context, b is the DNA radius (1 nm) and ξ is the concentration dependent correlation length [30] of the DNA polymer. The search time, which is defined as the average time for one DNA-binding protein to find one specific site, is related to the rate constant as $\tau = Vk_a^{-1}$ for a system of volume V. In order to predict the search time from the model, one needs to know the diffusion constant on DNA (D_1), residence time on non-specific DNA (t_R), and the effective diffusion constant combining diffusion in the cytoplasm and on DNA (D). We demonstrate below that these parameters can be measured by various single molecule methods.

4.1. Sliding on DNA

Although the 1D-diffusion along DNA, the central part of facilitated diffusion, has been inferred from in vitro binding assays since more than 40 years ago, it is only recently that direct observation has been made with single molecule fluorescence approaches [4,13]. Using flow-stretched DNA under microscope, individual fluorescently tagged proteins can be observed diffusing along the DNA. By tracking the fluorescent spot with a sensitive CCD camera, one can measure the 1D-diffusion constant as the ratio of mean-square displacement over time. In vivo, such diffusion measurement remains difficult because the DNA is highly condensed.

The in vitro sliding assay applied to a variety of DNA-binding proteins –transcription factors, RNA polymerases, and DNA-repair enzymes – has shown that the 1D-diffusion constant is always two orders of magnitude smaller than the 3D-diffusion constant. This is due to the hydrodynamic constraint that the protein's translational and rotational motions must be coupled in order to keep the same charged surface towards the helical DNA. It should be stressed that the vast difference between the 1D- and 3D-diffusion constants is the key feature in facilitated diffusion. As a direct consequence, the 3D-diffusion is responsible for rapid sampling through the cell, and the 1D-diffusion is responsible for the redundant search near the target site.

Not all the quantities obtained by the in vitro sliding assay can however be applied to the situation in vivo. For instance, the sliding distance before dissociation depends on the dissociation rate constant, which is sensitive to the ionic strength. The typical salt concentration used for in vitro assays is often much lower than that in the cytoplasm in order to observe the diffusion for longer period of time. Therefore, the in vitro measured sliding distance is typically an overestimate of what is observed in vivo. Fortunately, the 1D-diffusion constant is not sensitive to the ionic strength, although some exceptions exist [21]. We determined the 1D-diffusion constant of LacI to be 0.046 $\mu m^2/s$, or 46 nm^2/ms.

4.2. Effective diffusion in the cell

In vivo, one can also observe the search process in action by tracking individual TFs diffusing through the nucleoid. In vivo single molecule tracking is an emerging tool to probe the kinetics of biomolecules. To do so, a temporarily localized fluorescent molecule is imaged as a diffraction-limited spot, whose center accurately reflects the location of the molecule [8,34]. A trajectory of a single molecule can then be recorded by imaging the same molecule over time and space. Such techniques have been used extensively to study slowly moving objects, in intracellular cargo transport [22,27] and for membrane-bound proteins [14]. In order to track a TF rapidly diffusing and searching for its target, the imaging time has to be so short that the TF appears temporarily localized. As described above in the search time measurement, the fluorescence of the diffusing TF is normally spread all over the cell at an imaging time between 100 ms and 1000 ms. However, because the molecule transiently binds to nonspecific DNA, where the 1D-diffusion is very slow, it is temporarily localized during the nonspecific residence time. Indeed, if one reduces the camera exposure time down to 5 ms (and the increase laser power correspondingly), clear diffraction-limited fluorescent spots are recovered in the cells with no specific binding. It therefore indicates that the nonspecific residence time is comparable to or shorter than 5 ms.

With the images of nonspecifically bound TFs, we made movies to analyze their diffusion across the nucleoid. Stroboscopic laser excitation is used to create short laser exposure with variable imaging speeds. The movies revealed that the motion is diffusive, with no apparent signs of anomalous diffusion above the 10 ms time scale, where a mixture of 3D-diffusion in cytoplasm and 1D-diffusion along DNA [8] is observed, with an effective diffusion constant:

$$D_{eff} = (1-f)D_3 + \frac{fD_1}{3} \quad (2)$$

The weighting factor f is the fraction of time the TF spends along DNA while searching for the target. So far D_1 and D_{eff} have been measured, but in order to know f, we also need to measure D_3, the 3D-diffusion constant in the cytoplasm.

4.3. Diffusion in the cytoplasm

When it comes to measuring the pure 3D-diffusion of LacI in the cytoplasm, we can remove the small N-terminal DNA-binding domain and study the protein motion without interaction with DNA. However, single molecule tracking of the YFP-labeled protein is not possible in the absence of nonspecific DNA-binding, because the exposure time would need to be reduced to below 1 ms. Such a short exposure time requires a laser power that saturates the fluorophore, causing poor signal-to-background ratio. One alternative option to measure the 3D diffusion constant is FRAP (fluorescence recovery after photobleaching) [11], a method that employs a high intensity laser pulse to photobleach fluorescence in one spot of a cell, and the fluorescence recovery time can be used to measure the diffusion constant. The obvious drawbacks are that the methods can only be used for high copy number proteins and that the results depend on fitting to a geometrical model of the cell. Instead we opted for fluorescence correlation spectroscopy (FCS) [11,24]. In FCS a low intensity laser beam is focused into the cell, from which the fluorescence is collected at high time resolution by a confocally aligned avalanche photodiode (APD). The fluorescence time trace contains information about how long time individual molecules stay in the excited volume. The diffusion constant can be obtained from the photon-by-photon correlation time and from the size of the confocal volume. The FCS measurement of the YFP-labeled LacI dimer showed that the 3D-diffusion constant is 3 $\mu m^2\, s^{-1}$, which is one order of magnitude higher than the effective diffusion constant, and two orders of magnitude higher than the 1D-diffusion constant along DNA.

4.4. Predicted search time

With the three diffusion constants, we can now calculate the fraction of time spent on DNA based on Eq. (2). This fraction is ~90%, in agreement with the early population-averaged estimate by von Hippel and co-workers [19]. At thermodynamic steady state, this fraction is also equal to the ratio between the dissociation and association rates for nonspecific TF–DNA interaction. Assuming that that association is diffusion limited, we estimate that the dissociation rate constant as ~1000 s^{-1}, and the nonspecific residence time as ~1 ms[8]. Together with the effective and 1D-diffusion constant, we use Eq. (1) to estimate the search time. The predicted search time is approximately 1 min per molecule per site. This is about six times faster than the measured search time. In order to remove the discrepancy an improved model was constructed [23].

5. Improved model with in vivo considerations

What is missing in the model? The original facilitated diffusion model was developed for the in vitro situation, with only two components – TFs and DNA. In living cells, however, many other factors come into play. A major factor is the high protein occupancy on the chromosomal DNA (Fig. 2). These other DNA-binding proteins would reduce the concentration of accessible non-specific DNA, partially overlap with the specific site, and block the free sliding length. Of these effects, the restricted sliding length has the most dramatic consequence on the search time [23]. Shorter sliding distance means smaller effective target size, and hence slower search speed. At the occupancy expected for *E. coli* (30%), the increase in search time is approximately a factor of 2.

Although target recognition can be sped up by increasing the number of TFs searching for the target, this strategy can however only be used for a selected number of proteins, since a general increase in the number of DNA-binding proteins would increase the crowding on the chromosome, reducing sliding distances which would result in longer search time. A clear prediction from the new model is therefore that the search time increases if the occupancy of the chromosome is artificially increased.

We also suggest that the effect of roadblocks can partly be circumvented by positioning other specific operator sites at some distance away from the operator site that is used for regulation. If the distance between the auxiliary site and the regulatory site is farther away than the average sliding distance, they will appear as independent search targets, and it is twice as fast to locate any one of them. Upon initial binding, rapid binding to the second site, such as by DNA looping [23], as compared to the initial search time will ensure that the overall binding time to the regulatory site will also be twice as fast. One reason that DNA looping by TFs is so common may therefore be that it speeds up target location. This can also be the reason that there are two auxiliary operators in the *lac* operon although only one is needed to from a strong loop.

6. Long search time helped understand bistable switch of lac operon

The single molecule measurement of the lac repressor search time has subsequently helped elucidate the molecular mechanism of the bistable switch in the lac operon [5]. It has been known that the bistability of the lactose utilization network in *E. coli* originates from the positive feedback in gene regulation-intracellular inducer molecules inhibit the lac repressor, allowing the production of the lac permease, which in turn promotes inducer uptake and further inhibits the repressor. At intermediate inducer concentration, two distinct levels of lac operon expression, basal and fully induced, coexist in an isogenic cell population. It is, however, unclear what triggers the cell to switch from basal expression to the fully induced state.

One natural hypothesis is that one molecule of permease is enough to induce the positive feedback and thereby the switching. However, by counting the number of permease-YFP molecules in single *E. coli* cells with single molecule sensitivity, it becomes clear that the basal expression level (greater than one per cell) of permease is not sufficient to induce the positive feedback. In fact, the threshold to switch is at hundreds of permease molecules in a cell. So, what is the rare event that can generate such a large burst of permease production?

The search time measurement outlined above argues that when a lac repressor completely falls off from the lac operon, the time it takes to repress the operon again is on the order of minutes, during which several rounds of transcription initiation can already occur. Therefore, the *complete* dissociation of the repressor could be the triggering event that generates a large burst of gene expression to push the number of permease molecules above threshold. Since the lac repressor is a tetramer that binds to two distant operator sites on a DNA loop, most dissociation events will be *partial*, instead of complete, with one of its binding domains still attached to the operator. Because of the spatial vicinity, the rebinding of a partially dissociated repressor would be much faster than that of a completely dissociated one, giving rise to a much smaller transcriptional burst, as seen in the basal expression level. In summary, the in vivo kinetics data suggests that the compete dissociation of the lac repressor is rare and can produce large amount of permease molecules, both fitting the criteria of the bistability switching event.

Indeed, when DNA looping is removed from the lac operon, making every dissociation event complete, every cell rapidly switches to the fully induced state upon induction [5]. Since DNA looping is responsible for retaining the repressor, this finding supports that complete dissociation of the repressor is the rate-limiting step in the phenotypical switch of the lac operon.

7. Future perspectives

Such single molecule approaches to in vivo kinetics are not limited to studying transcription factors. As long as the protein of interest either has transient interaction with stationary objects, such as binding to chromsomes and ribosomes, or diffuses slowly itself, such as membrane-bound proteins, the kinetics can be studied in great detail. For example, by tracking individual membrane-bound FtsZ, a prokaryotic cytoskeleton protein, Niu and Yu showed that a subpopulation of FtsZ diffuses in a helical-shaped region [28], indicating a previously unknown bacterial membrane organization. We believe that in the near future the method will continue to generate novel information on other important cellular components, such as ribosome cofactors and chromosome maintenance enzymes.

Although single molecule imaging has led to tremendous discoveries in bacterial cells, it remains difficult to achieve the same sensitivity in eukaryotic cells. A typical eukaryotic cell is two to three orders of magnitude larger than a typical prokaryotic cell. Whereas the thickness of a prokaryotic cell is about the same as the axial resolution of a high numerical aperture objective, the thickness of a eukaryotic cell is so large that the out-of-focus autofluorescence overwhelms the signal of a single fluorescent protein. To circumvent the high autofluorescence in axial direction, total internal reflection fluorescence microscopy (TIRFM) is often employed to reduce the excitation depth. In recent years TIRFM has had great success in studying membrane protein dynamics in eukaryotic cells [25,27]. However, because TIRFM is only applicable to objects located a few hundred nanometers within the coverslip, it cannot be used to image single fluorescent proteins inside a eukaryotic cell. To probe all cytoplasmic proteins and nuclear proteins, a novel technique will be required to achieve single molecule sensitivity.

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