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Direct observation and analysis of the dynamics of the photo-responsive transcription factor GAL4

Guruprasad Raghavan,[a] [c] Kumi Hidaka,[a] Hiroshi Sugiyama,*[a] [b] and Masayuki Endo*[a] [b]

Abstract: Here, we report the direct visualization of the dynamic behavior of transcription factor GAL4 with photo-switching functions (GAL4-VVD) in the DNA origami structure. Using high-speed atomic force microscopy (HS-AFM), we observed photo-induced complex formation between GAL4-VVD and substrate DNAs. Dynamic behaviors of GAL4-VVD, such as binding, sliding, stalling, and dissociation with two substrate DNA strands containing specific GAL4 binding sites, were observed. We also observed inter-strand jumping on two double-stranded (ds) DNAs. On a long substrate DNA strand that contained five binding sites, a series of GAL4-VVD/DNA interactions, including binding, sliding, stalling, and dissociation, could be identified while in interaction with the surface. We also found a clear difference in the movement of GAL4-VVD while sliding or stalling in the AFM images. Detailed analysis revealed that GAL4-VVD randomly moved on the dsDNA using sliding and hopping for rapidly search of specific binding sites, and then stalled to the specific sites for the stable complex formation. These results suggest the existence of different conformational mode of the protein for sliding and stalling. This single-molecule imaging system with the nanoscale resolution provides an insight into the searching mechanism used by the DNA binding proteins.

DNA binding proteins, including transcription factors, play an important role in gene expression and control of cellular functions. Transcription factors function as molecular switches to regulate transcription; this dynamic behavior involving DNA needs further elucidation in order to understand the switching mechanism. Direct observation of the transcription factors and enzymes has been carried out and several searching mechanisms for target sites, including sliding, hopping, intersegmental transfer, and three dimensional diffusion, have been proposed. [1] [2] [3] The dynamics of the DNA binding proteins during the search for target sites has been mainly studied using fluorescence microscopy at sub-micrometer scale resolution. [1] [2] [3] [4] However, the detailed observation of these proteins at nanoscale resolution has not been achieved. Binding of a transcription factor to specific DNA sequence is often regulated by the dimerization of homo- or hetero- DNA-binding domains, which is controlled by the interaction of two α-helices called leucine zippers. [5] One well-known transcription factor that functions via this type of dimerization is GAL4. [6] The presence of the Zn-finger moiety in GAL4 enables it to bind to DNA in a sequence-selective manner. The systems comprising GAL4 and its specific DNA sequence have been used for targeted gene expression in multiple model organisms. [7] [8] [9] Photo-controlled dimerization of the VIVID protein (VVD, obtained from the fungus *neurospora crassa*) [10] has been used to control spatiotemporal, targeted gene expression by the GAL4 system. [11] As the dimerization domain is essential for the binding of GAL4 to the specific DNA sequence, replacing it with the VVD protein that possesses a photo-controlled dimerization motif enables the optical control of the interaction between the protein and the substrate DNA (Figure 1a). [11]

Herein, we intended to directly observe the dynamic movement of transcription factor GAL4 by optically controlling its binding to the substrate dsDNA, with the goal of understanding the single-molecule behavior of GAL4. For the AFM observation, photo-controlled activation is advantageous because its binding to the substrate and initial state of interaction can be directly observed. We designed DNA nanostructures to study GAL4 binding to the upstream activating sequence of GAL (*UASG*) using photo-controllable VVD dimerization. Our group has been developed an artificial setup of target dsDNA bridges, namely, the DNA origami frame. [12] Using this system, the physical properties of the DNA strands, such as tension, rotation, and orientation, can be precisely controlled for biochemical and biophysical studies. [12] Earlier experiments have shown that the physical control of the double-helical DNA bridges affects the enzymatic reactions, such as DNA methylation, [13] base excision repair, [14] recombination, [15],[16], and Cas9-mediated DNA cleavage. [17] Along with studying this system at stable equilibrium, dynamics of the interaction can also be inferred from this setup while observing with high-speed AFM (HS-AFM). [12]

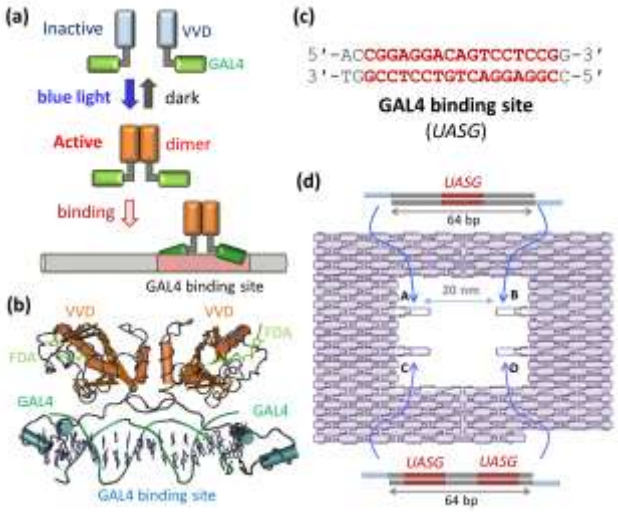

Figure 1. System for the direct observation of photo-responsive transcription factor (GAL4-VVD). (a) Illustration depicting DNA binding of GAL4-VVD controlled by photoirradiation. (b) Docked image of the structure of GAL4-VVD bound to ds DNA; FDA: flavin adenosine dinucleotide. (c) GAL4 binding site

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UASG (17 bp). (d) DNA origami frame for observation of the behavior of GAL4-VVD using two dsDNA bridges (64 bp). The DNA bridges on the top and bottom consist of single and double GAL4 binding sites (*UASG*), respectively.

To selectively control the dimerization of the GAL4, we engineered photo-responsive GAL4 (GAL4-VVD) by incorporating the VVD-dimerization protein, which acts as a photoswitch and responds to the blue light irradiation. [11] The dimerization domain at the C-terminus of GAL4 was deleted and replaced with the photo-responsive VVD domain. After protein expression with flavin adenosine dinucleotide (FAD) and purification, a recombinant GAL4-VVD was used for the experiments (Figure 1). First, we examined the DNA frame in a reaction solution using AFM. The substrate DNA strands were assembled into a DNA frame in a solution containing 20 mM Tris-HCl (pH 7.6) and 10 mM $MgCl_2$. The two target dsDNAs were connected to the DNA frame in >90% yield.

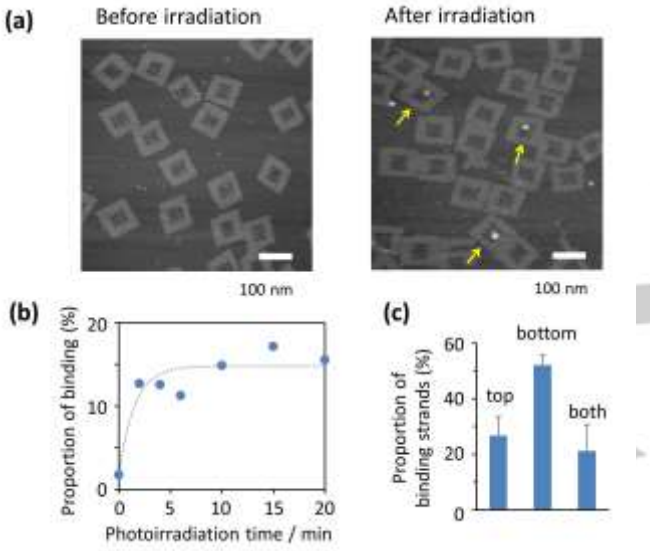

Figure 2. Observation of GAL4-VVD complex formation with substrate dsDNAs in the DNA frame. (a) AFM images of complex formation of GAL4-VVD before (left) and after (right) blue light irradiation. (b) Time-dependent complex formation by irradiation with blue light. (c) Proportion of the binding to the top and bottom DNA strand after 10 min of the blue light irradiation. Error bars show SD.

Then, we studied the time-dependent DNA-protein complex formation to quantify the irradiation time required to observe complexes based on the previous biochemical study. [11] We determined DNA-protein complex yield following irradiation for up to 20 min from AFM images. After irradiation with blue light (λ=450nm), the DNA-protein complex in the DNA frame structure was fixed by chemical crosslinking and the yield of the complex formation was calculated (Figure 2b). The binding yield was saturated at ~15% within 5 min. In addition, the yield of the complex formation with the top and bottom side of the bridges was compared. After 10 min of blue light irradiation, GAL4-VVD binding to the DNA bridges was 37% and 64% to the top and bottom sides, respectively, which was proportional to the number of *UASG* sites.

In an attempt to visualize and study the photo-controlled binding of *UASG*s present on the substrate DNA bridges, we observed dynamics of the GAL4-VVD/DNA interaction. During AFM scanning and blue light irradiation, we observed a series of protein/DNA interactions including binding, sliding, stalling, and dissociation (Figures 3 and S3, and Movie S1). Binding of the GAL4-VVD to the DNA strands and subsequent sliding were observed (Figure 3a,b). Additionally, dissociation of the bound GAL4-VVD was observed (marked with blue arrows, designated as A, Figure 3c). Furthermore, GAL4-VVD sliding along the DNA bridges and jumping between DNA bridges were also observed, before GAL4-VVD stably bound to its cognate sequence (marked by yellow and red arrows, designated as B, Figure 3c). Since the binding of the GAL4-VVD occurs only as a dimer, inter-strand jumping could occur when the DNA bridges were in close proximity, possibly because of Brownian motion of the bridges. In addition, the weak interaction between the protein and mica surface during inter-strand jumping may prevent the diffusion into the solution. Using the DNA frame cavity, distance between two dsDNA bridges can be controlled, which increases the chances of observing the inter-strand jumping. These results showed that the substrate dsDNAs in the DNA frame placed on the mica surface were recognized by GAL4-VVD initiated by photo-induced dimerization, and the basic behaviors of the DNA binding protein could be observed using this system.

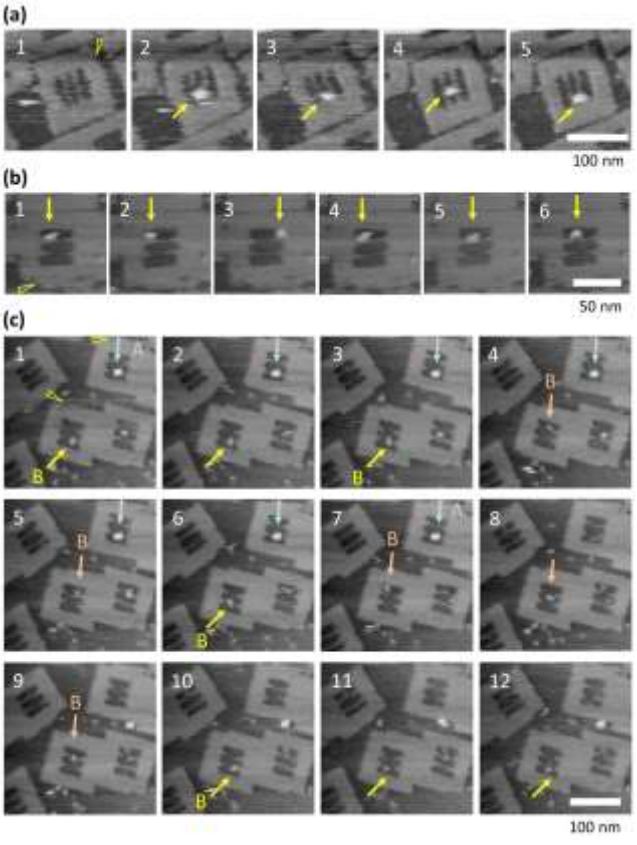

Figure 3. Direct observation of the movement of GAL4-VVD during blue light irradiation. (a) Binding and sliding of GAL4-VVD. (b) Sliding of GAL4-VVD. (c) Stalling and dissociation of GAL4-VVD (as indicated by blue arrows labelled as A). Sliding and jumping of GAL4-VVD between two dsDNA bridges (designated as B arrows). Yellow and red arrows indicate the positions of GAL4-VVD on the top and bottom strands, respectively. Successive AFM images were obtained at 0.2 frame/s. Yellow triangles represent the orientation markers of the DNA frame.

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Next, we used a DNA frame with a wide cavity to observe a series of GAL4-VVD behaviors. A long substrate dsDNA bridge (158 bp), containing five *UASG* sites, was incorporated into the DNA frame (Figure 4a). To observe binding of GAL4-VVD, blue light was irradiated on the AFM stage. After 10 min of irradiation, binding of the GAL4-VVD to the DNA was observed with a yield of ~15% (Figure 4b). AFM images were scanned at 0.2 frame/s. During the photoirradiation, various behaviors such as binding, sliding, stalling, and dissociation were observed using this system (Figure 4c, Figure S4, Movie S2). GAL4-VVD could move along the dsDNA and mainly stalled and slid on the *UASG* sites. Interestingly, GAL4-VVD always slid on one side of the dsDNA bridge. This means that the GAL4-VVD moved on the surface of the dsDNA and did not move along the groove.

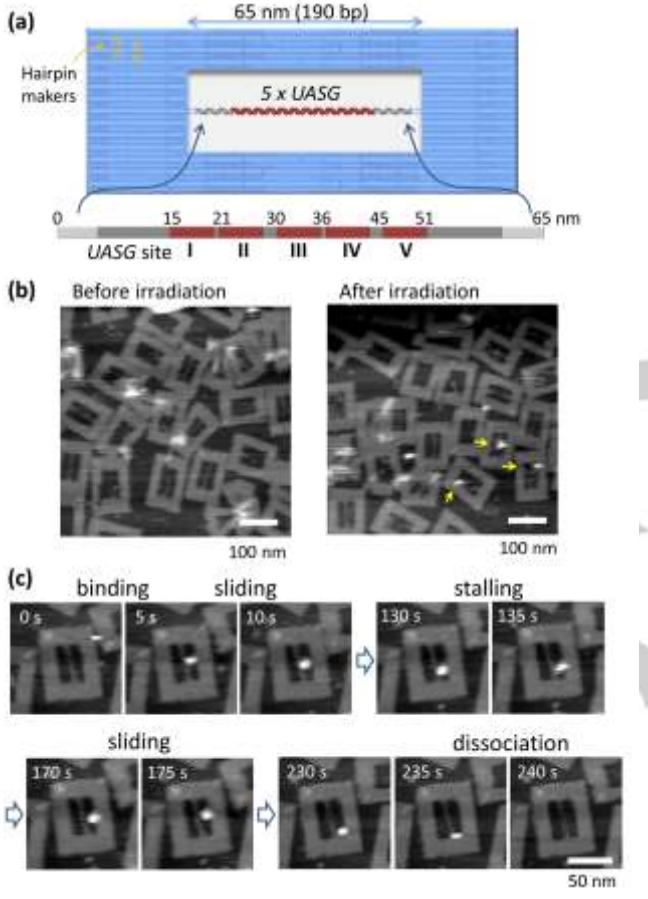

Figure 4. Direct observation of GAL4-VVD on the dsDNA bridge in the DNA frame. (a) Illustration depicting the DNA frame with dsDNA containing five *UASG* sites (158 bp) and linkers at both the ends for connection with the DNA frame. (b) AFM images of binding of the GAL4-VVD before (left image) and after (right image) 10 min of blue light irradiation. (c) Time-lapsed HS-AFM images of the single GAL4-VVD behaviour showing binding, sliding, stalling, and dissociation.

Next, we observed the long-time sliding of GAL4-VVD along the dsDNA bridge in details by scanning at 0.2 frame/s (Figures 5 and S5, and Movie S3). GAL4-VVD moved randomly and slid along the dsDNA bridge without detachmentment (Figures 5a), showing that the sliding occurred in the nanospace. We also observed that GAL4-VVD moved on one side of the dsDNA bridge, indicating that it weakly interacted with the mica surface. The kymograph analysis revealed that sliding of GAL4-VVD was random along the dsDNA bridge. GAL4-VVD moved on the five consecutive *UASG* sites without being tightly bound to these sites for 230 s (frame 46), and then decelerated its movement and fluctuated around site I (Figure 5b). In addition, long distance transfer such as fast sliding or hopping along the dsDNA occurred. [1] [2] [3] We also observed multiple traces of GAL4-VVD in the same AFM image. The dsDNA bridge in these AFM images were perpendicular to the scanning direction of the AFM. This means that the GAL4-VVD moved along the dsDNA bridge in the same direction as that of the movement of AFM scanning, and thus the AFM probe multiply contacted the sliding protein during the scanning. It can also be inferred that the sliding of the protein was much faster than AFM scanning speed used in the experiment. The positional frequency of the GAL4-VVD in the five *UASG* sites along the dsDNA bridge during the sliding is summarized in Figure 5c; it indicates no specific preference to any of the five individual *UASG* sites.

Furthermore, we analyzed another series of the GAL4-VVD movement whose kymograph is shown in Figure 5d (Figure S6 and movie S4). We clearly observed the distinct difference in the behaviors of GAL4-VVD such as the sliding (5-175 s) and stalling (180-320 s) before and after frame 35. During the sliding, the fast movement of GAL4-VVD on the dsDNA bridge was observed, in which multiply scanned images of the protein were also observed. During the stalling, the movement of GAL4-VVD was clearly supressed. The GAL4-VVD was observed to have bound to the *USAG* sites, in which this protein (8 nm in length) fully occupied the 17 bp *UASG* sites (~6 nm). The overall 1D diffusion coefficient in the sliding was calculated to be $D = 6.7 \times 10^{1}$ nm^2 s^{-1}. Due to the interaction with the mica surface, the 1D diffusion coefficient obtained in this experiment was two orders of magnitude smaller as compared to the reported values for the DNA binding proteins in solution. [1] We also observed the fast sliding or hopping, in which GAL4-VVD moved more than 100 bp/frame. For the long distance transfer, hopping is advantageous, because proteins can rapidly move along the dsDNA for long distance via dissociative transfer to search for a specific site. [1] [2] [3] Two Zn-finger DNA binding domains work asymmetrically to realize the rapid transfer during the sliding. [18] In the kymograph shown in Figure 5d, two different modes of GAL4-VVD conformations and their switching could be suggested. During the stalling, two DNA biding domains in recognition mode were used for the complex formation at the specific site and for subsequent relocation within these sites (as observed after frame 36). On the other hand, during the sliding movement, DNA binding domains with a conformation suitable for searching would have been used for the sliding (as observed in frames 1-35), similar to that observed for the other Zn-finger protein.[1] [19] By visualizing the movement of the GAL4-VVD at the nanoscale resolution in the DNA frame, these two distinct modes viz. searching mode and recognition mode, could be identified.

In conclusion, we have directly observed the photo-responsive transcription factor GAL4-VVD in the DNA nanostructure at nanoscale resolution using HS-AFM. The photo-controllable dimerization system can be utilized for single-molecule imaging to initiate binding of GAL4. Using optical control,

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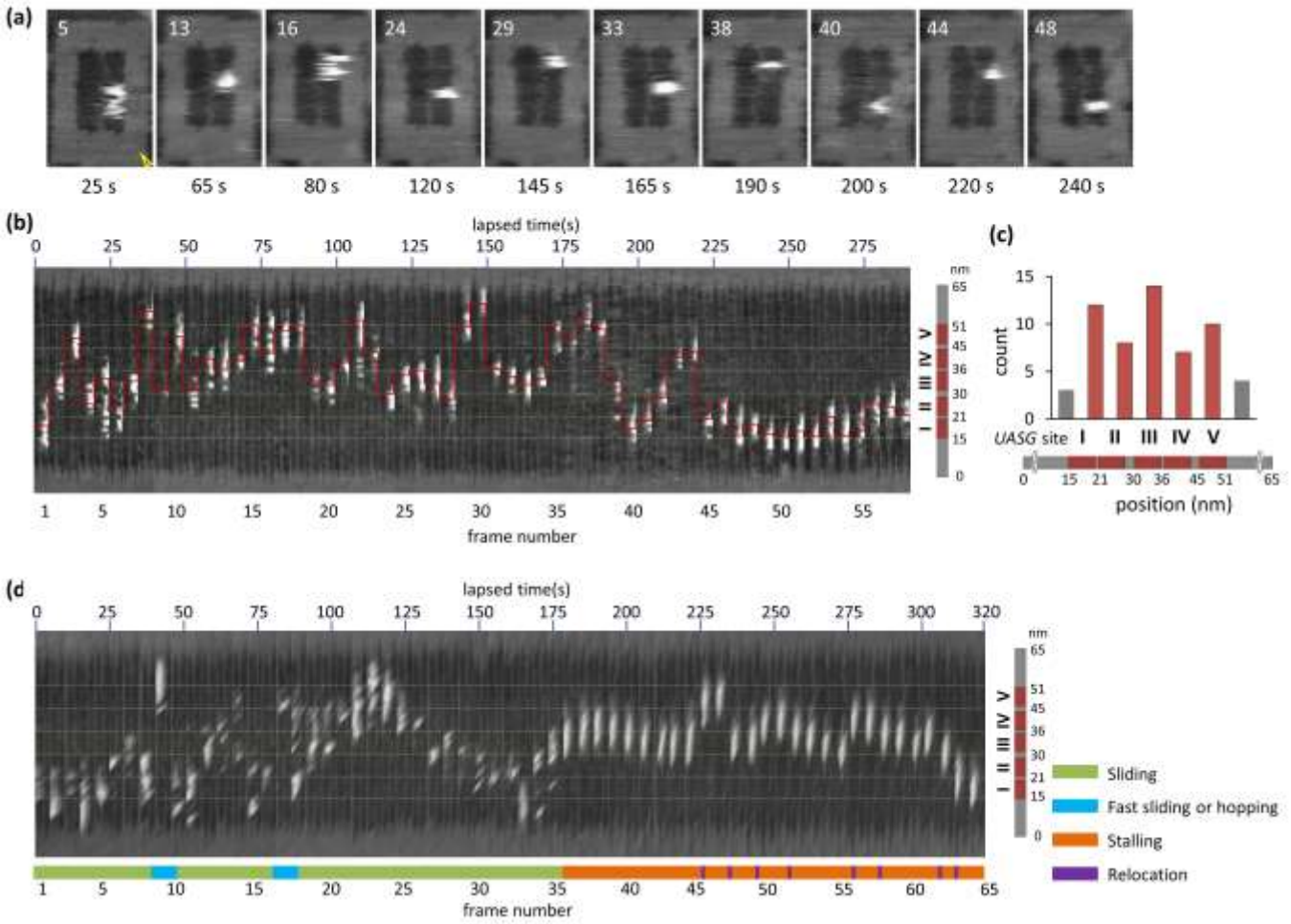

Figure 5. Direct observation of GAL4-VVD sliding on the dsDNA bridge containing five GAL4 binding sites (5×*UASG* sites) in the DNA frame. (a) GAL4-VVD movement on the substrate dsDNA in the DNA frame at various times. Images were obtained every 5 s (0.2 frame/s). Frame number is indicated in the images. Yellow triangle represents the orientation marker. (b) Kymograph of the movement of GAL4-VVD on the dsDNA containing five *UASG* sites. (c) Summary of the positions of GAL4-VVD at the five *UASG* sites. (d) Kymograph of the movement of GAL4-VVD on the dsDNA containing five *UASG* sites, obtained from the different series of HS-AFM images given in Figure S6.

a series of the behaviors of GAL4-VVD such as binding, sliding and stalling on dsDNA; inter-strand jumping between two dsDNA bridges and dissociation were clearly visualized using HS-AFM. In addition, sliding and stalling of GAL4-VVD on the multiple binding sites were visualized for relatively long time period without dissociation, and the binding mode was estimated by visualization and analysis of its movement along the dsDNA. This nano-sized single-molecule observation system can provide a solution to estimate and characterize a series of the behaviors of DNA binding proteins and enzymes before and during the recognition and actual process of catalysis.

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Keywords: DNA origami • transcription factor • photo-control • single-molecule observation• high-speed AFM

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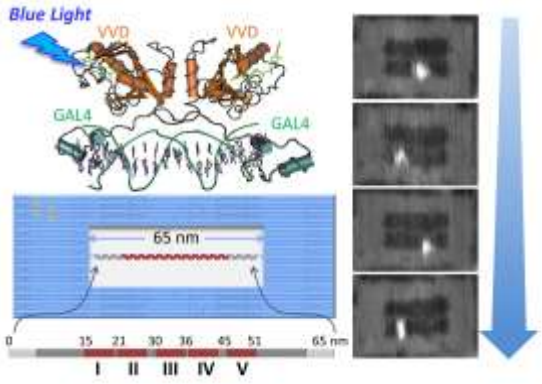
We demonstrate the direct observation of the dynamic behavior of the photoresponsive transcription factor GAL4 (GAL4-VVD) in the DNA origami structure using high-speed atomic force microscopy (HS-AFM). A series of movements of GAL4-VVD, including binding, sliding, hopping, stalling, and dissociation were observed under blue light irradiation. GAL4-VVD was observed to randomly slide on the dsDNA and stall at specific binding sites using different modes for rapid searching.



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