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Direct AFM Visualization of the Nanoscale Dynamics of Biomolecular Complexes

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Abstract

High-speed AFM (HS-AFM) is an advanced technique with numerous applications in biology, particularly in molecular biophysics. Developed as a time-lapse AFM technique for direct imaging fully hydrated biological molecules, HS-AFM is currently capable of visualizing the dynamics of biological molecules and their complexes at a video-data acquisition rate. Spatial resolution at the nanometer level is another important characteristic of HS-AFM. This review focuses on examples of primarily protein-DNA complexes to illustrate the high temporal and spatial resolution capabilities of HS-AFM that have resulted in novel models and/or the functional mechanisms of these biological systems.

INTRODUCTION

Atomic Force Microscope (AFM) belongs to a family of microscopes termed Scanning Probe Microscopes (SPM). Unlike optical and other microscopes that utilize primarily the diffraction phenomenon of an electromagnetic field, SPM instruments provide topographic images of surfaces by scanning them with a sharp probe. SPM began with the 1982 invention of the Scanning Tunneling Microscope (STM) by Binnig and Rohrer.¹ This invention was revolutionary in the material sciences due to its capability of visualizing individual atoms on a crystal surface. The inventors Binnig and Rohrer were awarded a Nobel Prize in physics in 1986 for their fundamental discovery. Interestingly, in that same year, one of these Nobel Prize winners, G. Binnig, published a paper entitled “Atomic Force Microscope,” in which AFM was described for the first time.² AFM applications in biological systems followed this invention, and this nanoimaging technique heralded new prospects in molecular biology for applications to study DNA and protein-DNA complexes.

The sample preservation concern typically associated with electron microscopy, widely applied before the invention of AFM was mitigated by a gentle sample preparation methodology. Moreover, AFM is capable of imaging samples in fully hydrated states and can do so by scanning in aqueous solutions. Therefore, in addition to acquiring static structural data at the nanoscale level for DNA and protein-DNA complexes, AFM can also visualize the conformational dynamics of these systems. Numerous reviews are available, including textbooks describing the application of AFM for biological systems (*e.g.*, refs. 3–6). This review focuses on the application of time-lapse AFM using high-speed AFM (HS-AFM) to study protein-DNA complexes. Importantly, HS-AFM is capable of direct visualization of the conformational dynamics of DNA, a number of protein-DNA complexes,

and protein oligomers at a sub-second data acquisition rate. Notably, the use of AFM to study the dynamics of membrane proteins is described in a number of recent papers,^{7–9} and therefore, this topic is omitted from this review given the other detailed sources as well as to focus on protein-DNA complexes.

Operation of Time-Lapse AFM

One of the most attractive features of AFM for use in biological applications is its ability to acquire images in aqueous solutions. In this AFM mode, the sample is imaged in a fully hydrated state, at the surface-liquid interface, and the dynamics of the sample are concurrently visualized after the acquisition of a set of consecutive images. This mode is termed time-lapse imaging. Given the nanometer range of AFM resolution, the dynamics of molecules, or the interaction of the molecules, can be visualized. Importantly, imaging in liquid eliminates the effects of capillary forces, a major factor complicating imaging at gentle conditions and decreasing the spatial resolution of AFM.¹⁰

Reliable visualization of molecules by using AFM requires their dynamic interaction with the surface via weak bonds. For example, imaging DNA dynamics requires weak DNA binding, thus permitting the molecule to move relatively freely at the surface. Therefore, the sample preparation procedure must be adjusted to reconcile these conflicting requirements. With regard to sample preparation, the most widely used approaches are cation-assisted methods for AFM imaging of DNA-protein-DNA complexes and mica surfaces.^{11–17} Our group pioneered different approaches based on the preparation of AFM substrates by chemical functionalization of primarily mica and glass surfaces (reviewed in^{18–23}). Our initial approach utilized 3-aminopropyltriethoxysilane (APTES), leading to the AP-mica procedure. Initially, the only drawback of AP-mica was that AP hydrolysis in water complicates the time-lapse AFM studies in aqueous solution. The problem was eliminated by the development of a procedure that uses 1(3-aminopropyl)silatrane (APS), which is resistant to the hydrolysis and polymerization at neutral pH, thus enabling AFM imaging, including time-lapse AFM studies, for a wide range of protein-DNA complexes.^{5; 10; 24; 25}

In the early time-lapse AFM studies, direct observation of fibrin self-assembly in aggregates was initially performed.²⁶ Following this early work, single-molecule AFM imaging was applied to study various phenomena such as observing how RNA polymerase moves along DNA,^{17; 27–30} characterizing local and global dynamics of supercoiled DNA,^{21; 31–33} directly visualizing the Holliday junction branch migration,^{21; 34} observing chromatin dynamics,^{23; 35} identifying conformational dynamics of membrane proteins (reviewed in^{36–38}), and detecting receptor dynamics of single molecules in the membranes of living cells.^{38; 39}

While novel properties of molecular systems were discovered with these time-lapse studies, these experiments also highlighted a unique set of problems associated with the time-lapse methodology. The joint effort of the Hansma and Bustamante groups in 1994 demonstrated the ability of time-lapse AFM to image the assembly process of RNA polymerase-DNA complexes.²⁸ Kasas *et al.* visualized the sliding of the polymerase along DNA⁴⁰ and showed that the diffusion of RNA polymerase along DNA constituted a mechanism for the location

of an accelerated promoter. Time-lapse experiments by Guthold *et al.*⁴¹ showed that, in addition to sliding, an enzyme could jump from one site to another. The real-time visualization of enzymatic degradation with a nuclease was performed in the paper by Bezanilla *et al.*¹⁵ Further, Argaman *et al.* were able to accelerate the data acquisition time by the use of the phase-imaging mode, making it possible to visualize DNA replication by Sequenase™ in real time.⁴²

Time-lapse AFM has recently been applied to study the structure and dynamics of mononucleosomes (nucleosome core particles [NCP]).^{43–46} The NCP sample was deposited on APS-mica and imaged with the omission of the drying step. Figure 1a illustrates the imaging frames of the dynamics of one selected NCP particle with ~two DNA turns. Initially, the DNA arms slightly unwrap (frames 1 → 2); the unwrapping process is more evident in frame 3. The NCP retains its geometry over three frames in a row (frames 3, 4, 5). Between frames 5 and 6, the NCP loosens and unwraps again in frame 6. It remains unchanged in frame 7 and finally undergoes full dissociation in frame 8. The length measurement (Fig. 1b) shows that the nucleosome dissociation is accompanied by unwrapping of both DNA flanks; although during the final stages (frames 6 and 7), the process is asymmetric with substantial elongation of one arm compared to another. Similar observations led to the conclusion that the nucleosome undergoes spontaneous dissociation, although the specific path can vary from particle to particle.

These findings demonstrating the spontaneous dissociation of nucleosomes seemingly contradicts numerous biochemical and biophysical studies of nucleosomes performed with the use of ensemble techniques, including X-ray crystallographic studies that describe the particles as having well-defined structural characteristics. One dramatic difference between ensemble and single-molecule studies is the concentration of nucleosomes. Single-molecule studies are typically performed at a much lower nucleosome concentration. Importantly, it was found that nucleosomes at nanomolar concentrations are unstable and undergo spontaneous dissociation, thus requiring the use of detergents to stabilize them (*e.g.*, ref. ⁴⁷ and references therein).

High-Speed AFM

AFM images are reconstructed through point-by-point probing of the surface topography. This operation is typically performed in the range of one scan line per second (scanning frequency of 2 Hz). Therefore, the acquisition of an entire image comprised of 512 lines (512 data points per line) takes approximately 4 minutes. For dry samples, this relatively slow speed of the AFM operation is a matter of inconvenience but, in fact, is not a serious bottleneck in the entire data acquisition process. However, a slow data acquisition rate of AFM is a serious drawback when time-lapse AFM is applied to image the majority of biological processes. For example, the translocation of RNA polymerase along DNA occurs on the second timescale; therefore the observation on the minute timescale reveals only a small percentage of events.

The first fast-scanning AFM was developed by the group of AFM co-inventor C. Quate,⁴⁸ in which an integrated active actuator was used to achieve a high-scanning speed. However, the stiffness of active probes inhibited implementing this concept in the imaging of soft

biological systems. The breakthrough in the development of the high-speed (i.e., HS) AFM for biological applications was made in 1996 after achieving the concept of a short cantilever, proposed by the group of P. Hansma.⁴⁹ The initial HS-AFM design made by the Hansma group provided data acquisition at a rate of 0.5 frames/sec, which was 100-times faster than the rate of a conventional AFM.^{50; 51}

The short cantilever concept was further developed by T. Ando in his first design of the HS-AFM instrument, which was capable operating at a sub-second image acquisition speed.^{52–55} Key features of the HS-AFM instrument realized in the Ando design are as follows: (1) small cantilevers with a resonant frequency above several hundred kHz and a small spring constant, (2) a high-speed scanner with a resonant frequency that matches the cantilever characteristics, (3) active damping techniques to suppress mechanical vibrations of the scanner, and (4) fast feedback control. Moving forward, the current HS-AFM instrument can capture images at the video rate without significant disturbance of weak biomolecular interactions.⁵⁶

The drive amplitude issue is especially important in HS-AFM due to the elevated scanning speed. Therefore, operating the instrument at a low-drive amplitude received careful attention in the current instrument design that operates with amplitudes at an order of magnitude less than traditional AFM.

The analysis of the effect of the tip on the sample for HS-AFM was performed by Miyagi *et al.*⁴⁵ HS-AFM operates in a *tapping* mode; therefore, it is important to consider the effect of the following two major factors: hitting the sample with an oscillating tip and displacement of the sample during scanning. The energy transferred by the tip to the sample is proportional to the square of the oscillation amplitude. HS-AFM operates with an amplitude of ~1 nm, and therefore, the estimates by Miyagi *et al.*⁴⁵ demonstrate that the overall energy induced by the tip is equivalent to the increase of temperature by only ~9.6 K. Given that this energy is distributed across the entire system, including water molecules in the vicinity of the sample, this effect is low. Another effect of the tip is the displacement of the sample. Because of the high oscillation frequency, the time during which the tip contacts the sample is ~100 nanoseconds (ns); therefore, during this period of time, the sample stage moves in the x-direction only by 0.16 nm. This value is comparable to the size of an atom; thus, the lateral displacement of the sample by the scanning tip is negligible.

A potential dragging effect of the tip was investigated by Kobayashi *et al.*,⁵⁷ in which the displacement a DNA segment was measured along the scanning direction of the tip, as well as perpendicular to the tip, of the HS-AFM instrument. A difference has not been found between the two directions, suggesting that the lateral force applied to the sample does not essentially increase with the increasing of scan rate. Additional studies were conducted by Suzuki *et al.*⁵⁸ in which the authors calculated the elastic bending energies and fluctuations of single-molecule conformations to assess the effect of the probe.

The sections below briefly describe the results of several papers in which interesting biological problems were targeted using this unique nanoimaging approach. The HS-AFM instrumentation is under a constant development, as evidenced in recent publications

describing the latest developments in this area.^{8; 59–61} These articles also describe current limitations of HSAFM in terms of temporal and spatial resolution and scan size, and discuss prospects for future improvements in HS-AFM instrumentation.

Nanoscale Visualization of Myosin V Translocation Along the Actin Fibril—

Myosin V belongs to a family of adenosine triphosphate (ATP)-dependent motor proteins that play key roles in muscle contraction and other motility processes. Myosin V is critical in vesicle movement from the center of the cell to the periphery and functions as a dynamic tether, retaining vesicles and organelles in the actin-rich periphery of cells. To accomplish its function, myosin V ‘walks’ along actin filaments with a ~36 nm step towards the positive end of the filaments. This knowledge was primarily obtained by the use of single-molecule optical imaging and optical-trap probing techniques that demonstrated the processive hand-over-hand movement of the motor, coupled with hydrolysis of ATP (*e.g.*, ref. ⁶²). Previous imaging studies of proteins using electron microscopy, x-ray crystallography, and nuclear magnetic resonance have only produced static images of the protein. By using HS-AFM, Ando and colleagues were able to simultaneously record the structure and dynamics of myosin V molecules moving along actin filaments.^{56; 63} In AFM images, myosin V initially appears in a V-shape, attached to the actin filament at both legs. Over time, one of the legs detaches from the filament, whereas the other leg remains bound to the same position on the filament. During next step, the initially dissociated leg binds to the actin filament, thus restoring the V-shape of myosin V.

The above experiments were performed in the presence of excess unbound streptavidin molecules hitting of which decelerated the myosin-walking process and allowed the investigators to visualize the process by using the temporal resolution of the instrument. The results are in line with the model of myosin walking and therefore represent the first direct visualization of this important biological process at a molecular-level resolution. These studies also revealed a number of dynamic properties of myosin V, such as the junction flexibility, but also highlighted future HS-AFM studies.⁶⁴

Site-Search Process of Site-Specific DNA-Binding Proteins—The interaction between distant DNA regions is the key step in fundamental genetic processes such as site-specific recombination, integration, excision, and inversion of specific DNA regions within genomes,⁶⁵ as well as V(D)J recombination process required for the antibodies assembly.⁶⁶ This interaction is controlled by a specialized protein, or protein complex, responsible for forming a site-specific protein-DNA synaptic complex (*i.e.*, synaptosome). In fact, the formation of a synaptosome is a general phenomenon not limited to site-specific recombination systems. For example, a large family of DNA restriction enzymes form synaptosomes to facilitate further site-specific DNA cleavage.^{67–70} The mechanisms behind the formation of synaptosomes elicit several questions. For example, how does the protein locate both specific sites? It appears that it finds one site and then searches for another. How does the search process occur? If the two sites are located in one DNA molecules, DNA loops are formed. Does looping create structural constraints? This question was addressed by Gilmore *et al.*,⁷¹ in which the interaction of EcoRII restriction enzyme belonging to the synaptosome family of site-specific DNA proteins was visualized with HS-AFM.

The time-lapse experiments by Gilmore *et al.* showed that EcoRII slides and dissociates/associates, employing 3D and 2D diffusion while searching for the first site. Unexpected behavior was found when EcoRII captured two DNA sites, with one of the sites being specific, leading to the formation of the loop. Frame 2.5 s of Figure 2a shows this initial looped structure, with the protein appearing as a bright blot at the intersection of the strands. The images following this illustrate the dynamics of the complex formed by the DNA loop. It is seen by the comparison of frames 5.0 s and 7.5 s that the loop increases over time, and the long arm decreases in size. This process continues until the last image (frame 20.0 s), which shows a considerable increase in the size of the loop.

This process was also analyzed by contour length measurements (Fig. 2b). The analysis reveals that the short arm 1 remains constant over the observation period, and its length corresponds to the position for the EcoRII-specific-binding site at 100 base pairs (bp) from the DNA end, suggesting that the protein remains specifically bound at this recognition site the entire time. The length of arm 2 fluctuates, with its shortening starting after the 5 s interval, when the loop size is between 283 bp (96 nm) and 312 bp (106 nm). At about 6 s, the loop length begins to increase, while the length of arm 2 decreases, showing that translocation occurs along arm 2. After 10 s, translocation stops; this position corresponds to the location of another specific-DNA site for EcoRII, as validated by length measurements equaling 34 nm or 100 bp from the end. During this process, the loop becomes as large as 610 bp. Thus, the translocation process covers a distance of about 300 bp (102 nm), with a mean rate of about 30 bp/s (10.2 nm/s).

A number of conclusions emerged from these studies. First, the search process is such that EcoRII remains bound at one DNA-recognition site and searches for another site by threading the DNA filament through another DNA-binding pocket of the enzyme. This occurs until the enzyme finds the second recognition site and subsequently forms a stable synaptic complex. Second, although there are fluctuations in the loop size, the loop itself gradually increases given that the formation of larger loop is entropically favorable. Third, the protein translocation is not accompanied by its rotation around the DNA helix, considering that DNA supercoiling within the loop would accompany protein translocation were this the case. This finding suggests that during translocation, the protein slides along the DNA filament potentially via small ‘hops.’

Dynamics of Centromere Nucleosomes—Centromeres are specialized segments of chromosomes that aid in chromosomal segregation after DNA replication. A kinetochore is a protein complex that interacts specifically with the centromere of a chromosome to allow for the separation of sister chromosomes into daughter cells. If the centromere becomes damaged or removed, the chromosomes segregate randomly. This suggests that centromeres contain specific structural characteristics that allow for their selection by kinetochores. However, the structural details of centromeres and the mechanisms underlying this recognition process by kinetochores remain unclear.

It is known that, at the nucleosomal level, all centromere nucleosomes contain modified H3 histones (CENP-A; *e.g.*, ref. ^{72, 73}). The crystallographic data for CENP-A and H3nucleosomes⁷⁴ can be used to generate models for nucleosome arrangements of both

types of nucleosomes.⁷⁵ Traditional nucleosomes have entry and exit flanks of DNA crossing at $\sim 90^\circ$ (Fig. 3, scheme a); whereas in CENP-A nucleosomes, the flanks are almost parallel (0°) because the wrapped DNA is shorter by 26 bp (Fig. 3, scheme b). The arrangement of these units into arrays leads to the well-known zig-zag model for H3-nucleosomes (Fig. 3, scheme c), whereas CENP-A nucleosomes form a compact linear array (Fig. 3, scheme d). Does this nucleosomal arrangement lead to different higher-order structures for centromeres and regular chromatin? This is a question to be answered in future studies. However, specific considerations must first be taken into account.

Publications^{76; 77} challenge the finding that CENP-A nucleosomes have decreased heights. However, studies performed on mononucleosomes⁷⁶ and arrays⁷¹ using both types of H3 histones did not reveal a height difference. These papers provide a number of explanations for these discrepancies, including potential problems with AFM experiments. As one explanation, the authors suggest that CENP-A nucleosomes have increased dynamics compared those of regular nucleosomes. Therefore, the structural studies should be accompanied by dynamic studies, which require the use of approaches employing single-molecule biophysics capable of characterizing transient states of biological systems. HS-AFM is an attractive application for such studies considering HS-AFM is capable of visualizing the structural features at the nanometer resolution.

In 2018, Stumme-Diers *et al.* applied HS-AFM to study the dynamics of CENP-A mononucleosomes.⁷⁸ Specifically, mononucleosomes containing human CENP-A instead of the H3 histone were reconstituted on a 423 bp DNA substrate containing the 147 bp Widom 601-positioning sequence^{79; 80} inside the DNA fragment with 122 bp and 154 bp flanks of nonspecific DNA sequences. Nucleosomes assembled on this template were localized within the 601 motif, similar to canonical nucleosomes assembled with H2, H3, and H4 histones (*i.e.*, H3nucleosomes). AFM images of CENP-A nucleosomes were qualitatively similar to those for H3nucleosomes, although quantitative analysis revealed a wrapping efficiency with ~ 1.5 turns for CENP-A nucleosomes compared to ~ 1.7 turns for H3 nucleosomes. This finding is in a perfect correlation with the X-ray model for CENP-A nucleosomes.⁷⁴ Additionally, the distribution of the length of wrapped-DNA CENP-A nucleosomes was bimodal, and high-resolution imaging revealed the appearance of DNA loops near nucleosomes that had not been seen in images of canonical nucleosomes. This feature was confirmed by time-lapse HS-AFM imaging, as illustrated in Figure 4.

The images shown in Figure 4 visually illustrate that the DNA loop grows gradually (frames 1–3), until it reaches a size of ~ 90 bp, as seen in frame 4. Next, the loop shrinks (frames 5–7), becoming again small (~ 20 bp) in frame 8. To characterize this process, images of all 100 frames were analyzed; the complete dynamics can be seen clearly in movies in the publication.⁷⁸ Further, quantitative analysis of these images via contour measurements of the nucleosome arm was performed to characterize changes in the DNA wrapped around the core. Similar HS-AFM studies have been performed with canonical nucleosomes and did not reveal comparable dynamics with such extensive loop formation.^{45; 47; 81; 82} HS-AFM imaging identified the following two additional new features of CENP-A nucleosome dynamics: translocation and transfer of nucleosomes, as briefly described below.

CENP-A nucleosomes are capable of long-range translocation. This property is illustrated graphically in Figure 5, in which the position of the nucleosome is shown at one end of the DNA template depending on the time (frame number). This graph, obtained by compiling over 500 frames, demonstrates that the initial translocation takes place when the nucleosome is partially unwrapped, and the DNA moves around the core until it reaches the end of the DNA substrate. This ‘forward’ translocation (Fig. 5a) moves the DNA a total of ~180 bp, with two important stall events along the path. The first stall event is after the DNA moves ~25 bp, and the second is after it moves ~75 bp. Then, the DNA moves ~70 bp to the end of the DNA strand, where it remains bound for few hundred frames. Next, the DNA begins a reverse translocation of ~180 bp, back to its starting position (Fig. 5b). There are also stall events at distances ~60 bp, ~52 bp, ~40 bp, and ~25 bp (Fig. 4b).

Figure 6 illustrates the event in which the core particle transfers from one DNA molecule to another. Figure 6a shows a set of a few of these AFM images. Initially, two nucleosomes are shown in the same scanning area. One nucleosome unwraps, leaving a free-DNA substrate (frame 2), which is later approached by the second nucleosome core particle (frame 3). This nucleosome core particle becomes associated simultaneously with two DNA molecules (frame 3 and 4), eventually transferring its core to the free-DNA substrate. Figure 6b schematically visualizes this process. These two events of DNA looping are distinct features of CENP-A nucleosomes that have not been observed in canonical H3 nucleosomes.

The findings described above suggest that the CENP-A nucleosome core retains the integrity of the histone core, enabling it to translocate along the DNA and transfer from one DNA template to another. Indeed, direct comparative experiments performed by Stumme-Diers *et al.*⁷⁸ demonstrated that after unwrapping, canonical nucleosomes dissociate into histones, whereas the CENP-A nucleosome remains as a large particle. It was also hypothesized that this novel property of CENP-A allows it to stay associated with H2A, H2B, and H4, thereby permitting rapid nucleosome reassembly following mitosis, transcription, or replication-induced eviction. Furthermore, the stabilization of fully wrapped CENP-A nucleosomes by CENP-C,⁸³ along with the intrinsic stability of unwrapped CENP-A cores, may contribute to its longevity on the chromatin fiber, thus contributing to the cell-cycle independent ‘memory’ of centromeric chromatin over several cell cycles.⁸⁴

High-Resolution Imaging of HS-AFM and Nanoscale Dynamics of Proteins—

The major factor defining AFM resolution is the sharpness of the tip. As such, with an atomically sharp tip, atomic or even subatomic resolution can be achieved.⁸⁵ The dynamics of the sample are another factor defining the resolution. Accordingly, reliable atomic-level images of organic molecules can be obtained at temperatures near 0 K, thus enabling investigators to measure the length of interatomic bonds.⁸⁵ However, at ambient conditions, AFM resolution is limited to the nanometer range and, in addition to the tip geometry, depends on environmental conditions.¹⁰ Furthermore, if imaging is performed in air, hydration of the sample and tip cause complications. Specifically, when the AFM tip approaches the surface, a water bridge forms between the hydrated tip and sample. The high surface tension leads to capillary forces as large as a dozen nano Newton (nN).⁸⁶ Therefore, the sample can be dragged over the surface during scanning, deteriorating the imaging process. However, drying of the sample and imaging at low relative humidity helps reduce

the effect of capillary forces. Alternatively, the capillary effect can also be reduced if scanning is performed in a vacuum, but a vacuum is not very attractive for imaging biological samples. At the same time, there are no capillary forces if the imaging is performed in aqueous solution, and given the fact that imaging in aqueous solutions is the ideal way to preserve biological samples, imaging in liquids is very appealing for biological studies.

Indeed, images of DNA with the highest resolution have been obtained in aqueous solutions.^{87; 88} Furthermore, high-resolution images were obtained for individual DNA molecules⁸⁸ rather than tightly packed DNA molecules.⁸⁷ Such high-resolution images are often obtained with HS-AFM instruments with the use of tips created by electron beam deposition followed by sharpening using a plasma-etching methodology.^{53; 56} A few examples illustrating the application of HS-AFM to study the dynamics of protein-DNA complexes with high resolution, as well as proteins assemblies, are given below.

Dynamics of Cas9-Substrate Complexes—Clustered Regularly Interspaced Short Palindromic Repeats/protein-9 nuclease (CRISPR/Cas9) is one of the most widely used microbial adaptive immune systems responsible for locating and cleaving target DNA, both in natural and in artificial CRISPR/Cas systems (*e.g.*, ref. ⁸⁹). The Cas9 protein is a multifunctional protein consisting of six domains. Crystallographic data have provided the atomic structure of Cas9^{89; 90} and suggest the mechanisms behind the function of this protein result from a highly dynamic feature of the protein. This feature, detailed in the next paragraph, has been demonstrated by the use of high-resolution HS-AFM,⁹¹ and a few images in Figure 7 illustrate the major finding, discussed below.

The set of consecutive frames selected in Figure 7a shows that initially the Cas9 protein adopts a compact conformation, although it is far from a smooth globular shape that reflects the domain structure of Cas9, as revealed in crystallographic studies.^{89; 90} Over time, the shape and contrast of individual segments change, suggesting the protein undergoes intramolecular dynamics. The geometry and dynamics of Cas9 also change as it binds to RNA. According to the set of images in Figure 7b, the protein adopts a clear, bi-lobed geometry, which remains stable for the same timeframe as the free protein. Graphs in Figure 7c illustrate this difference, in which correlation coefficients between sequential AFM images are calculated. There is a large fluctuation for the free Cas9 protein, whereas the value remains constant for the Cas9-RNA complex. Additionally, the investigators examined the process underlying the dynamics of DNA cleavage accomplished by Cas9.⁹¹ They found fluctuations in the nuclease domain of Cas9 in complexes corresponding to active and non-active states of the enzyme. Overall, these results not only revealed dynamic properties of Cas9 and the role of substrate in these dynamics, but also highlighted capabilities of the HS-AFM methodology for characterizing such large, macromolecular systems as CRISPR/Cas9.

Interdomain Dynamics of APOBEC 3G Protein—APOBEC3 proteins are cellular cytidine deaminases with important roles in mammalian innate immune responses.⁹² Among them, major attention has been given to APOBEC3G (A3G), which restricts the replication of HIV-1, the hepatitis B virus, retrotransposons, and other DNAbased parasites.⁹³ A3G is a two-domain protein. Its N-terminal domain (NTD) interacts with nucleic acids and Vif, and

its C-terminal catalytic domain (CTD) carries out deamination activity.^{94; 95} A key first step toward a detailed understanding of the functional activity of A3G is determining its atomic structure. Yet, the high-resolution atomic structure of the full-length A3G remains undefined, primarily due to oligomerization and precipitation of A3G at concentrations required for crystallization.^{96; 97} In order to characterize the interdomain dynamics of A3G, time-lapse HS-AFM imaging has been applied.⁹⁸

To ensure that the dynamics of the A3G monomer were followed, data were collected for the A3G monomer that dissociated from the complex formed between the A3G dimer and RNA.⁹⁸ These data were acquired by continuous scanning in liquid over the selected area, with a rate of 398 ms/frame; all frames were assembled as movies, available in the paper by Gorle *et al.*⁹⁸ A few selected frames illustrating the dynamics of A3G are shown in Figure 8a. Frame 1 shows A3G in a slightly elongated globular shape, which becomes extended in frame 15 and then returns to the initial shape in frame 22. To characterize the globular shape of the A3G, shown in Figure 8b, the ratio was calculated for two orthogonal parameters, d_1 and d_2 , defined in the inset “i” of Figure 8b. Measurements of $d_1:d_2$ ratio were performed for over 250 frames. These data are presented in Figure 8b, inset “ii”. The graph shown in Figure 8b demonstrates that 84% of the time A3G has a compact, globular shape that fluctuates between slightly elongated ($d_1:d_2 \sim 1.1$) and an ellipsoid ($d_1:d_2 \sim 1.7$). In the remaining 16% of cases, the A3G monomer adopts a clear dumbbell shape, as shown in Figure 8c. To characterize the dumbbell shape of A3G, the parameter d_3 was used, defined as the distance between the maxima of the peaks at the height of the cross-section of the A3G dumbbell (Fig. 8c, inset “iii”). Analysis of the dynamics of the dumbbell shape of A3G obtained from three separate movies, resulting in a histogram of d_3 distribution, are shown in Figure 8c (inset “iv”). The data set was approximated by a Gaussian distribution, with a maxima at 4.5 nm, indicating that the distance fluctuates between the CTD and NTD domains of A3G. Taken together, data in Figure 8 show that the A3G monomer exists in the following two forms: a globular form (~84% of the time) and a dumbbell form (~16% of the time), and can dynamically switch from one form to the other.

Details of the A3G structure and dynamics were further revealed in the same paper by building an atom-level model of the full-sized A3G via computational modeling and docking of the CTD and NTD domains of A3G based on available structures, followed by microsecond-long molecular dynamics simulations.^{98,97,94,92,92,93,94,93,83} Analysis of protein shapes in the computed ensemble of A3G structures reaffirmed the A3G monomer exists predominantly in globular and dumbbell forms (Fig. 9). These forms are characterized by the parameter d_3 , defined as the distance between centers of masses of the NTD and CTD. Figure 9 shows the histogram distribution of d_3 distances for the whole A3G ensemble, where d_3 values range from 3.2 nm to 4.6 nm. While most of the A3G structures are found to exist in the compact globular shape (here, taken to be $d_3 < 4.2$ nm), a significant population assumes a dumbbell-like form (here, taken to be $d_3 > 4.2$ nm). Thus, the simulations revealed a highly dynamic feature of the A3G monomer that can lead to extended conformations in which two domains are separated by distances as large as 4.5 nm. The results of computational modeling including the population of the dumbbell conformation are fully in line with experimental HS-AFM results.

It has been suggested that the interdomain dynamics contribute to the A3G search for the catalytic binding pose,⁹⁸ especially considering the nucleic acid-binding residues are located on different A3G domains and at solvent-exposed surfaces that can be spatially distant. Furthermore, the interdomain dynamics could facilitate the process of viral RNA packaging into the HIV virion, which is aided by interactions with A3G.

Nanoscale Dynamics of Non-Structured Proteins Revealed by HS-AFM—

Studies during the recent decade reveal a class of proteins termed intrinsically disordered proteins (IDP), which are highly dynamic and do not contain segments with well-defined secondary structures such as α -helices or β -strands.⁹⁹ Initially this family was limited to amyloid-type proteins, such as amyloid- β proteins and peptides and α -synuclein (α -syn), but recent data has shown that this class of proteins is abundant. IDP-type segments are found many proteins, including Cas9.¹⁰⁰ Given a highly dynamic feature of IDP proteins or IDP segments within structurally organized proteins, their characterization requires special methods.⁹⁹ Single-molecule biophysics approaches are very attractive given their ability to characterize transient states of macromolecules. Furthermore, recent studies have demonstrated the applicability of time-lapse HS-AFM, for which a few examples are reviewed in this subsection.

The early application of HS-AFM for IDP-containing proteins, as previously described,^{53; 101} revealed that such segments can adopt an extended conformation, and they appear in AFM images as flexible filaments. The mechanical properties of IDP segments of chromatin transcription factor FACT (i.e., facilitates chromatin transcription) have been obtained from these studies. Similar observations were made by Zhang *et al.*,¹⁰² in which the dynamics of full-sized α -syn monomers, as its transiently assembled dimeric forms, were studied. For background, α -syn is the major component of the intraneuronal inclusions called Lewy bodies, which are the pathological hallmark of Parkinson's disease. The protein α -Syn is capable of self-assembly into many different forms, such as soluble oligomers and fibrils. Even though attempts to resolve the structures of this protein have been made, current knowledge lacks a detailed understanding of these structures and their relationship with the different aggregation steps. This understanding is of principal interest for providing insights into the pathogenic mechanism of Parkinson's disease.

Imaged with HS-AFM, the spherical conformation of α -syn appears initially as a globular shape (Fig. 10, frame 0 s) that transitions into a one-tailed state (Fig. 10, frame 13.8 s), followed by fluctuations between globular and one-tailed conformations (Fig. 10, 16.8 s and 26 s). These events are then supplanted by the transition to a two-tailed structure (Fig. 10, frame 27.4 s). The conversion from the globular shape directly into two-tailed conformations has also been observed (Fig. 10, frames 27.4 s, 33.6 s, 62.6 s and 100.2 s). Interestingly, also observed was the transition between the different conformations with tails and extended structures with compact terminal segments (two heads, Fig. 10, frame 102.2 s) (Fig. 10, frames 125.6 s, 129.6 s, 130.4 s). Eventually, the monomer was found to adopt an extended conformation (Fig. 10, frames 136.6 s and 161.2 s). Computational simulations revealed that the α -syn monomer consists of several tightly packed, small helices. The tail-like protrusions are also helical with a small β -sheet, acting as a "hinge." Monomers within dimers have a large interfacial interaction area and are stabilized by interactions in the non-

amyloid component (NAC) regions. Furthermore, the dimer NAC region of each α -syn monomer forms a β -rich segment. Moreover, NAC regions are located in the hydrophobic core of the dimer.

By treating the protruding tails of the α -syn monomers as a polymer on a 2D surface, one can obtain the persistence length (P) of the polymer.¹⁰² This analysis yielded a value of $P = 2.9$ nm. This value is approximately five-fold less than that previously reported for a similar intrinsically disordered chromatin transcription (*i.e.*, FACT) protein,¹⁰¹ suggesting that the tails of α -syn monomers are more flexible, and their mechanical properties are different.

Nanoscale Dynamics of Oligomers Assembled by Amyloid Beta Protein—

Conformational plasticity of amyloid proteins is the key feature that enables them to spontaneously assemble into aggregates and leads to the development of such neurodegenerative diseases as Alzheimer's, Parkinson's, and Huntington's, among other neurological disorders.^{103,107} The most widely studied amyloid aggregates are fibrillar assemblies in which well-defined segments of the protein adopt the β -rich conformation to provide a highly-ordered fibrillar morphology to the aggregates (*e.g.*, ref. ¹⁰⁴). Although amyloid fibrils are the major components of plaques in the brain, numerous data reveal that oligomeric forms of amyloid aggregates, rather than fibrils, are neurotoxic. This suggests that structural studies of these types of aggregates are needed for the development of efficient treatment and diagnostic strategies for neurodegenerative diseases involving protein aggregation. However, data regarding the structures formed during the early stages of the aggregation process have been unavailable, thus highlighting the urgent need to discover the structural properties of these species.

Amyloid protein oligomers exist transiently, complicating the use of traditional structural techniques to study their characteristics. However, the recent implementation of single-molecule imaging and probing techniques capable of examining transient states have enabled certain properties of these oligomers to be characterized.^{107; 108} Here, the results of a recent paper¹⁰⁹ are reviewed briefly, in which HS-AFM was applied to reveal the nanoscale dynamics of oligomers of amyloid beta (A β) 42. To make advancement this possible, the oligomers were photochemically cross-linked so they could be separated according to size and then purified.^{110; 111} These A β 42 oligomers remain neurotoxic even after the cross-linking, making them an attractive experimental system for biomedical studies.

Oligomers were prepared in 10 mM sodium phosphate (pH 7.4) and imaged with HSAFM, without drying.^{35; 45} Figure 11a shows eight frames of a representative trimer to illustrate the dynamics of the A β 42 oligomer. Frame 2 shows an elongated, globular, single-blob shape. Figure 11b shows a plot illustrating the changes in length and width of the trimer during the time-lapse experiment. Figure 12a shows a set of 10 representative images from the time-lapse imaging of the A β 42 pentamer oligomer. This assembly displays a spherical shape initially, as shown in frames 1 and 91. The oligomer remains mainly in this structure up to frame 204, except in a few cases in which the molecule becomes more elongated (frame 101). Frame 204 illustrates a substantial structural change that appears to be followed by the development of a large protrusion (frame 210). This elongated structure remains

stable for a number of frames before collapsing back into a compact, single-blob shape (frame 248). After this, the pentamer shows smaller protrusions (frame 254) but generally remains in its single-blob form (frame 257). Figure 12b shows 3D views of five of the structures of the pentamer selected from the set in Figure 12a. The 3D view of frame 1 clearly shows the compact, single-blob structure, whereas the 3D projections of frames 210 and 218 display an elongated structure with the protruded, tail-like feature attached to the initial blob. Height measurements along the long-axis of the pentamer structures show that the pentamer in frame 1 produces a symmetric profile (Fig. 12c), whereas the pentamer in frame 210 yields an asymmetric profile. Interestingly, the maximum height in this profile is less than that shown for frame 1. This is due to extension of a part of the oligomer from the compact shape to the elongated one, resulting in a drop in the height value. Frames 218 and 224 show the presence of two peaks, which correspond to the two bulges present in those two particular structures. The relatively symmetric profile from frame 248 confirms the return of the oligomer to the initial single-blob shape (Fig. 12c).

The structural transition of heptamers follows the same pattern as pentamers. The heptamer can transiently adopt three blob-like structures. In this complex and dynamic process, the initial compact, one-blob assembly transitions into the two-blob structure. This dynamic assembly undergoes rearrangements of the three-blob morphology that occur *via* the formation of two-blob-like structures with unequal blob sizes. In comparison, the compact, elongated, ellipsoidal morphology of trimers does not change, except for occasional elongation of the ellipsoid.

These observations suggest that A β 42 oligomers are highly dynamic. The authors hypothesize that dimers and trimers are the basic units of oligomers and are the direct products of high-order oligomer dissociation. Therefore, the entire heptamer appears to be in continuous rearrangement between a compact shape and multi-blob morphologies comprised of dimers or trimers. Spontaneous unfolding of higher-order A β 42 oligomers into the more stable dimers or trimers has important biomedical implications. The results of HS-AFM studies indicate that oligomers as large as heptamers are in a dynamic equilibrium with their dimer and trimer building blocks. This suggests that targeting these two types of assemblies would be sufficient for blocking higher-order assembly. If the therapeutic agents were antibody-specific for unique epitopes on the dimer and trimer, this therapy also would allow immune clearance of the resulting immune complexes.

CONCLUSIONS

Although the development HS-AFM began almost two decades ago, biological applications for this technique are much more recent. Therefore, it is reasonable to place biological applications of HS-AFM starting at the year 2008, with the publication of a revolutionary paper by the T. Ando group,⁵³ which demonstrated the application of HS-AFM to study the dynamics of various biological systems. Additionally, in this paper authors provided a detailed description of an instrument they developed and built. The technique, due primarily to efforts of T. Ando, then became available to the AFM practitioners across the world.^{60; 112; 113}, Note the critical contribution to the HS-AFM development from the group of G. Fantner⁶¹, so the overall efforts made it possible for the major AFM manufactures to build

commercially available instruments. As a result, the HS-AFM instrumentation is currently available to the biomedical community.

Currently, AFM can be applied to various biological systems. Considering the wealth of data available, this review describes but a small portion of publications focused on the dynamics of isolated molecular systems, without yet touching upon published papers regarding the dynamics of membrane proteins.^{60; 112} In addition to topographic imaging, HS-AFM has been applied to probing interdomain interactions within proteins.¹¹⁴ Availability of the high-speed pulling instrument allows one to compare experimental data with Steered Molecular Dynamics (SMD) computational simulations to garner details on the rupture process at the atomic level. Future development of HS-AFM towards higher speeds⁶⁰ will help one approach experiments meeting the requirements of SMD; however, recent developments of alternative computational approaches have made it possible to model AFM experiments at pulling rates used in typical AFM-probing experiments.^{106; 115; 116} Still, in the opinion of the author, the broadest application of the time-lapse AFM mode, and HS-AFM in particular, is in the topographic mode of HSAFM. Note in this regard the recent publication of the T. Ando group in which the dynamics of the CRISPR/Cas9 system revealed by HS-AFM were combined with high-resolution Cryo-EM structuring of this system.⁹¹ This combination allowed the authors to further the knowledge of the mechanism of action for this complex system.

The combination of HS-AFM data with molecular dynamics simulations is another application of HS-AFM with a great potential. Molecular dynamics simulations are currently capable of providing atomic-level structural data for rather large systems. However, these simulations typically result in a set of structures; therefore, the selection of appropriate structure(s) is one of the problems with computational modeling. High-resolution structural data are often not available, so the need for alternative approaches is in high demand. As demonstrated,⁹⁸ HS-AFM can be a useful validation approach.

Last but not least, one should keep in mind that *in vivo* conditions are far from the test-tube/*in vitro* conditions. The intracellular compartment contains a large number of organelles and vesicles; thus, the majority of biological systems operate by being bound to these organelles and cell membranes. However, surface models for AFM, with various characteristics that mimic the structure of membranes and other organelles, can be designed. As such, AFM as a topographic technique is currently an ideal and advanced method for characterizing the dynamics of the complex molecular/biological machinery at conditions approaching those *in vivo*.

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Figure 1:

The two-step spontaneous unwrapping process of nucleosomes. (A) Consecutive AFM images of nucleosomes with a two-step unwrapping process, taken during continuous scanning in buffer. Each frame is 200 nm. (B) Dependence of arm lengths on the frame number. Each frame takes about 170 second to scan.

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Figure 2.

HS-AFM to study of the dynamics of EcoRII-DNA complexes. (A) Individual frames shown every 2.5 s illustrate EcoRII translocation. As the long arm gradually decreases, the loop length gradually increases. (B) The dependence of DNA length on time measured in 0.5 s intervals. As the long arm gradually gets shorter, the loop length gradually increases. The contour lengths of the entire molecule and the short arm have consistent values over the entire timescale. The translocation over the length of 300 bp occurs within 10 s.

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Figure 3.

Schematic structures of H3 and CENP-A mononucleosomes (a and b, respectively) and their arrangements into arrays (c and d, respectively). Histone cores are shown as red, and green circles demonstrate H3 and CENP-A assemblies, respectively.

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Figure 4.

Spontaneous looping of CENP-A nucleosomes visualized by high-speed AFM. A set of AFM images representing stages of the looping of DNA from the nucleosome complex; numbers 1—8 correspond to the location of the AFM frames in the time trajectory. The line in image three shows how the height-profile cross sections. In total, looping was observed for 10 nucleosomes from the 52 particles imaged.

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Figure 5.

Nucleosome translocation is reversible along a DNA substrate. (A) Images of the forward (1–4) and reverse (5–9) translocation of a nucleosome core particle. Forward movement of the complex appears to be achieved via a corkscrew motion of the DNA. The reverse movement is of a less-wrapped complex and ends up at about the same position on the DNA substrate as where it began. The white arrows in images 1 and 6 point to the arm length for which the contour length measurements are shown in (B), where the distance was measured from the end of that arm to the center of the core particle for every frame of the video. Black circles represent the data points and the red line is a moving median. Sliding was observed for a total of six particles from the 52 particles imaged.

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Figure 6.

The stable CENP-A histone core is capable of spontaneous inter-strand transfer. (A) HS-AFM images depicting the transfer of a partially wrapped CENP-A-containing histone core from one strand to another and depicted in the schematic (B). Image one shows that the parent strand (grey) on the left is partly wrapped with the histone core (blue), free from interaction with the acceptor strand (green) on the right (image and schematic 1). The interaction of the acceptor strand (image and schematic 2) soon leads to a histone/DNA complex containing both parent and acceptor strands (image and schematic 3). Within a second for this dual-substrate complex, the acceptor strand takes full control of the core, with the parent strand only partly interacting (image and schematic 4), until the acceptor/core complex is completely free from interaction with the parent substrate (image and schematic 5).

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Figure 7.

Sequential HS-AFM images of apo-Cas9 (a) and Cas9-RNA (b) on the AP-mica surface. The color (from black to white) corresponds to the height. The scale bars are 10 nm. (c) Time courses of correlation coefficients between the sequential HS-AFM images of apo-Cas9 and Cas9-RNA.

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Figure 8.

Conformational dynamics of A3G. (a) Selected frames from the Supplementary Movie 7. The size of the images is 40 nm. The scanning rate corresponds to 398 ms per frame. (b) A representative AFM image of the globular-shaped A3G monomer. Insert “i” provides the definition of d_1 and d_2 . Insert “ii” shows the plot of fluctuations of the $d_1:d_2$ ratio of A3G monomer calculated for ~250 frames captured. The mean value of the $d_1:d_2$ ratio is equal to 1.3 ± 0.3 . (c) A representative image of the dumbbell conformation. The figure was reproduced from Gorle et al. (2017), Copyright © 2017, with permission from the American Chemical Society.

Figure 9.

Computer model of A3G. Histogram distribution of distances between NTD and CTD domains, d_3 , for the complete docking ensemble of A3G structures. The histogram is separated into structures that acquire globular ($d_3 < 4.2$ nm; black rectangles) and dumbbell forms ($d_3 > 4.2$ nm; grey rectangles). Insets show examples of A3G in globular and dumbbell forms. A3G is shown in surface representation, whereas NTD is in blue, and CTD is in red. The figure was reproduced from Gorle et al. (2017), Copyright © 2017, with permission from the American Chemical Society.

Figure 10.

HS-AFM images of wild-type α -syn monomers. Selected frames show the structural transition of the α -syn monomer over time. The monomer starts in a globular conformation (0 s) and transitions into the following conformations: one-tailed (13.8s, 26.0 s, 33.6 s, and 125.6 s), two-tailed (27.4 s, 62.6 s, 129.6 s and 132.6 s), and extended conformations (130.4 s and 136.6 s). Interspersed with the other conformations, the monomer transitions back into a globular conformation (16.8 s and 100.2 s).

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Figure 11: Dynamics of the Aβ42 trimer. (A) Time-lapse images of a cross-linked Aβ42 [Phe10, Tyr42] trimer visualized by HS-AFM. The numbers in the images indicate the frame number. (B) Plot showing the changes in length and width of the trimer during the time-lapse experiment. The frames shown in the inset indicate the corresponding data points, which have been measured from those frames.

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Figure 12:

Dynamics of A β 42 pentamer. (A) Time-lapse images of a cross-linked A β 42 [Phe10, Tyr42] pentamer visualized by HS-AFM. The numbers in the images indicate the frame number. The pentamer remains spherical in the initial frames (1–91), except in few frames where it becomes ellipsoidal, as exemplified in frame 101. The elongated conformation of the pentamer is shown from frames 210–224. Frames 248–257 show the compact spherical conformation, with some protruding features, as shown in frame 254. (B) A 3D view of few selected frames. (C) The cross-sectional profile of the pentamer along the long-axis of the molecule. The numbers on top of both the 3D view and cross-sectional profile represent the corresponding frame numbers. *The figure was reproduced from Banerjee et al (2017), Copyright © 2018, with permission from the American Chemical Society*