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High-speed AFM imaging

Toshio Ando[1,2,3]

Proteins are dynamic in nature and function at the single molecule level. To achieve a straightforward and in-depth understanding of their underlying functional mechanism, we need to directly observe protein molecules at work at high resolution, without the use of protein-attached markers. To realize such objectives, high-speed atomic force microscopy (HS-AFM) has been developed and recently its capability has been fully established. This approach opens a new avenue to directly and closely observe individual molecules at submolecular spatial resolution and sub-100 ms time resolution. The captured molecular movies of proteins directly report and provide great insights into how the proteins function. Moreover, the very recent progress of HS-AFM technology has extended its use to the observation of dynamic cellular processes. In this article, I review imaging studies to show the innovative power and potential of this new microscopy.

Addresses
[1] Department of Physics, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan
[2] Bio-AFM Frontier Research Center, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan
[3] CREST, Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchi 332-0012, Japan

Corresponding author: Ando, Toshio (tando@staff.kanazawa-u.ac.jp)

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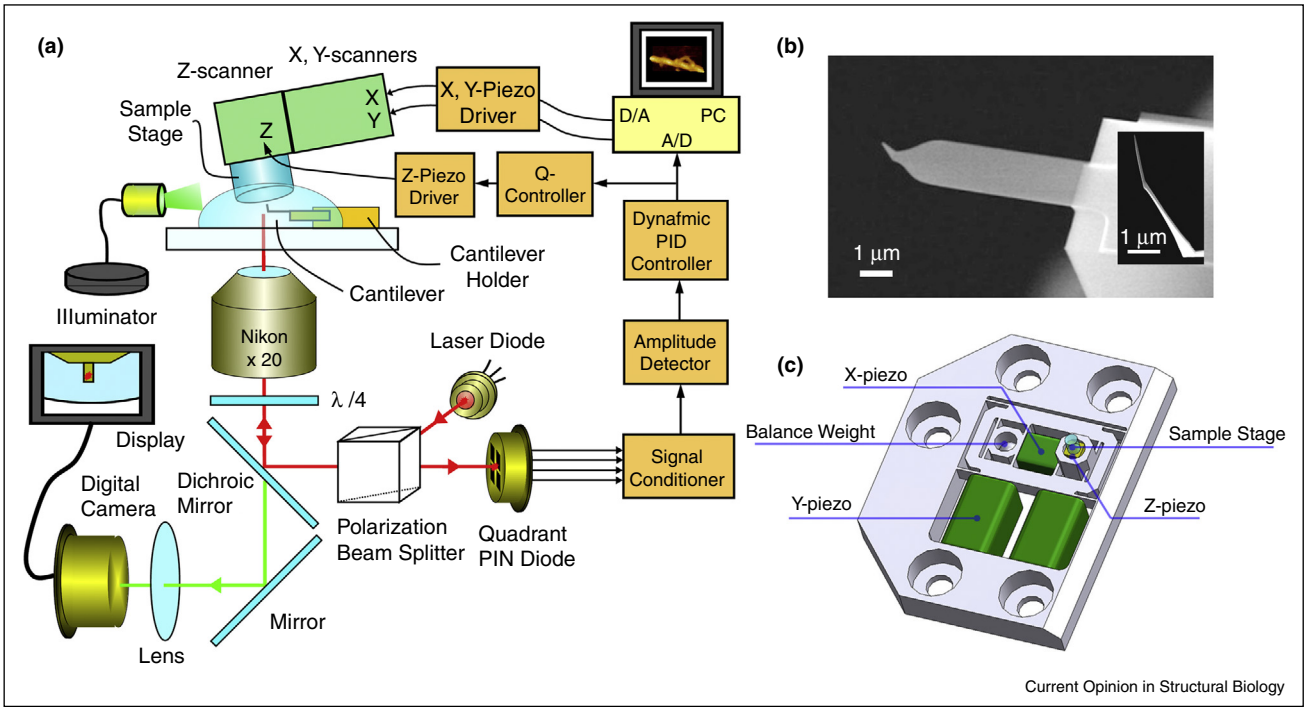
Introduction

The essential attributes of proteins, namely structure and dynamics, play central roles in their biological function. Structural biology has been successful over the past 60 years in solving the detailed three-dimensional structures of many proteins, by the use of X-ray crystallography, electron microscopy, and nuclear magnetic resonance spectroscopy methods. And yet, these methods are virtually ineffective in solving the second important attribute. To study the dynamics of proteins, single-molecule fluorescence microscopy [1,2] and optical trap nanometry [3] have been created and widely used over the last 20–30 years. However, these methods record the dynamic behavior of optical markers attached to protein molecules, and hence, the protein molecules themselves are completely invisible, even with superresolution fluorescence microscopy bypassing the diffraction limit [4]. Due to this indirectness of measurement, one has to infer how the protein molecules are actually behaving behind the recorded data. Thus, there have been no means that allow the simultaneous recording of structure and dynamics, preventing a straightforward understanding of how proteins function, except for proteins that serve rather as scaffolds than as functional entities. Under this adverse situation, a technique capable of observing both structure and dynamics of single protein molecules has long been desired, even with lower resolution than those of the conventional structural biology methods. To meet this desire, high-speed atomic force microscopy (HS-AFM) has been developed since 1993 [5,6], which was put into practical use around 2008 [7]. The imaging rate is now remarkably increased to higher than 10 frames per second (fps) and, importantly, the tip force acting on the biological sample does not disturb its function, while in spatial resolution HS-AFM is comparable to or slightly better than conventional AFM [8•,9]. This enhanced performance has enabled the direct visualization of dynamic structural changes and dynamic interactions occurring in individual molecules, which is currently not possible with other techniques. In fact, various applications of this microscopy have successfully revealed dynamic molecular processes of proteins, yielding remarkable findings and great insights into the functional mechanisms of the proteins (see comprehensive reviews [9,10••]). Moreover, this microscopy now allows the observation of dynamic molecular processes occurring on the surfaces of live bacteria [11•], specialized eukaryotic cells [12•] and probably isolated intracellular organelles, as well as dynamic processes occurring in eukaryotic cells [13].

HS-AFM system

HS-AFM typically employs the tapping-mode, in which a cantilever is oscillated in the *Z*-direction at or near its resonant frequency so that the tip intermittently taps the sample surface. In the HS-AFM system (Figure 1a), various devices, including cantilevers (Figure 1b), electronic circuits, the sample-stage scanner (Figure 1c), and the cantilever deflection detection system, are optimized for achieving high-speed performance [7,9]. For example, the cantilevers are much more miniaturized (6–12 μm long) than conventional ones to achieve high resonant frequencies in water f_c = 400 kHz to 1.2 MHz and small spring constants k_c = 0.1–0.2 N/m. In addition, a new feedback control technique capable of

Figure 1



Schematic illustration showing HS-AFM system and devices contained therein. **(a)** Overall arrangement of all devices contained in the HS-AFM system (reprinted with permission from Ref. [14•]. Copyright 2012 Nature Publishing Group). **(b)** Scanning electron micrograph of a small cantilever for HS-AFM. The inset shows an electron beam deposited amorphous carbon tip grown on the original tip. **(c)** Structure of a HS-AFM scanner for narrow area imaging (<1 μm × 4 μm).

maintaining weak tip-sample interactions and active damping techniques to suppress the scanner's mechanical vibrations have been implemented [7,9]. The speed performance of an AFM system is expressed by the bandwidth of feedback control f_B, defined by the feedback frequency at which $\pi/4$ phase delay occurs in tip-tracing of the sample surface. The most advanced HS-AFM system has achieved $f_B = 110$ kHz. The highest possible imaging rate R_{max} is a function of various parameters, including f_B, the scan range in the X-direction W, the number of scan lines N, the spatial frequency of the sample surface corrugation to be imaged $1/\lambda$, and the maximum possible phase delay in tracing the sample surface θ_m at which the resulting excessive force exerted by the tip does not disturb the biological function of the sample. R_{max} is expressed as $R_{max} = 2\lambda\theta_m f_B/(\pi NW)$ [8•,14•]. θ_m depends on the sample fragility and is typically $\sim\pi/9$ for proteins, according to previous imaging studies [14•]. For example, under the conditions $f_B = 110$ kHz, $W = 200$ nm, $N = 100$, $\theta_m = \pi/9$, and $\lambda = 10$ nm, R_{max} becomes 12 fps.

HS-AFM imaging of purified proteins

Thus far, various types of dynamic events occurring in protein systems have been visualized, as listed in Table 1: first, conformational changes; second, motor actions; third, diffusion and interactions in membranes; fourth, self-assembly processes; fifth, wiggling motion of and order–disorder transitions in intrinsically disordered proteins (IDPs); sixth, enzymatic reactions; and seventh, DNA–protein interactions. In some cases, molecular structures are not well resolved. Nevertheless, the visualized dynamic events inaccessible with other approaches have provided significant insights into the molecular processes, as exemplified in the imaging study of cellulase hydrolyzing crystalline cellulose fibers [36]. The visualization of interaction dynamics of membrane proteins allows the assessment of interaction geometry and energy in detail, as demonstrated [29,30]. The process of protein self-assembly generally contains various events and structures: the formation of nucleation clusters, multiple intermediates, multiple sequential and parallel growing pathways with different kinetics, and structural polarity and anisotropy. HS-AFM can show most, if not all, of these progressing events and growing structures in one video recording [31,32]. The visualization of thin and very flexible unstructured peptide chains allows the fast identification and characterization of the disordered regions in IDPs (Figure 2a) [33–35].

Thus far, the most striking results have been obtained in the observation of conformational changes of

Table 1

Various types of dynamic events of proteins visualized by HS-AFM

Conformational changes
- Bacteriorhodopsin responding to light [15–17]
- Rotary propagation of conformational changes over the β subunits of F_1-ATPase [18]
- ATP-induced channel pore dilation of $P2X_4$ receptor [19]
- ATP-induced height change of Ca^{2+} pump [20]
- Agonist-induced height change of NMDA receptor [21]
- Height change of acid-sensing ion channel in response to acidification [22]
- Straight-to-curving structural change of FtsZ polymers [23]
- ATP-dependent rotation of AAA+ chaperone p97 [24]

Motor action
- Walking myosin V on actin filaments [25]

Diffusion and interactions in membranes
- Interaction between aquaporin-0 tetramers in reconstituted eye lens fiber cell membranes [26]
- Diffusion of and interaction between bacterial outer membrane protein OmpF molecules in reconstituted membranes [27]
- Diffusion and fusion of vacancy defects in streptavidin 2D crystals formed on supported lipid bilayers [28]
- Membrane-mediated interaction between ATP-synthase c-rings [29]
- Interaction of bR trimers with bR crystal edges in purple membranes [30]

Self-assembly processes
- Amyloid-like fibril formation by lithostathine [31]
- 2D crystallization of sphingomyelin-specific pore-forming toxin on planar lipid membranes [32]

Dynamics occurring in intrinsically disordered proteins
- Wiggling and shortening/extension motion of disordered regions in FACT protein [33]
- Order–disorder transition of a disordered region in FACT protein and effect of phosphorylation [34]
- Wiggling and shortening/extension motion of a disordered region in CENP-T [35]

Enzymatic reactions
- Processive movement and traffic congestion of cellulase hydrolyzing crystalline cellulose fibers [36]

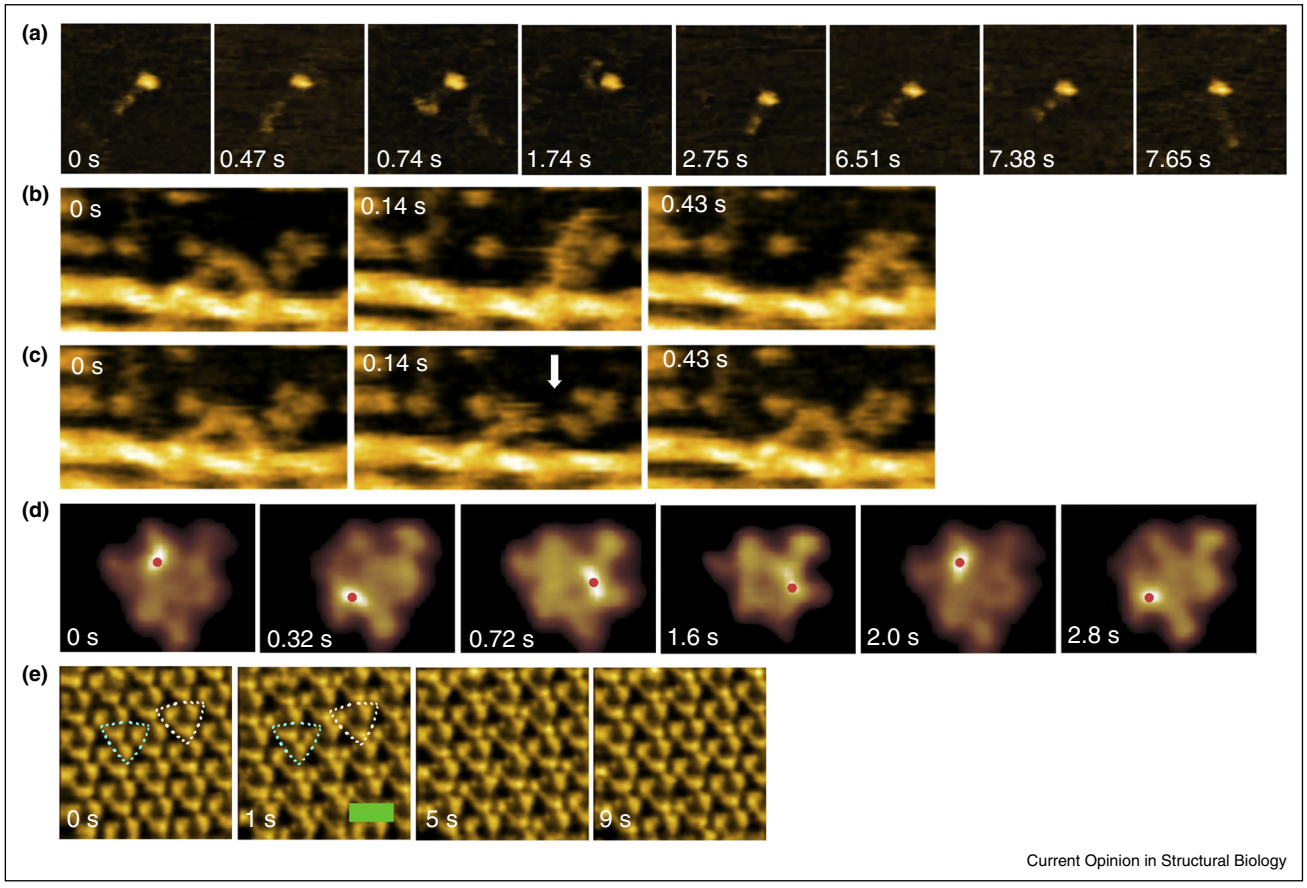
DNA–protein interactions
- One-step and two-step dissociation of ssDNA binding protein oligomer from ssDNA [37]
- Cleavage of DNA by type IIF restriction enzyme [38]
- Nicking enzyme-mediated unidirectional movement of DNA motor along DNA stator track [39]
- ATP-independent unwrapping of mono-nucleosomes [40]

Diffusion and interactions on live cell surfaces
- Diffusion of crowded porin molecules on live bacterial outer surface [11•]
- Water channel aquaporin-0 binding to and dissociating from junctional microdomains on the lens cell surface [12•]

bacteriorhodopsin (bR) [15–17] and F_1-ATPase [18] and motor actions of myosin V [25]. The dynamic behaviors of these proteins were visualized in unprecedented detail, leading to significant findings. This is partly because their dynamic actions are closely related to their biological functions, and therefore, the functions themselves are directly displayed on screen. A molecular movie of myosin V walking on actin filaments showed its forward movement with a ~36 nm stride in a hand-over-hand manner, rotational motion of the detached trailing head around the forwardly biased neck-neck junction (Figure 2b), foot stomp in the leading head (Figure 2c), and the lever-arm swing of the leading head spontaneously following the trailing head detachment from actin (Figure 2b). The foot stomp and the swinging lever-arm motion were observed for the first time by HS-AFM. HS-AFM movies of actin-bound myosin V captured in the presence of ADP or under nucleotide-free condition revealed other dynamic actions, which led to the clarification of the hand-over-hand walking mechanism and an important discovery concerning the usage of ATP energy. As exemplified in this HS-AFM study, HS-AFM imaging has the unique feature that multiple dynamic events can be detected simultaneously in one video recording. This feature makes a stark contrast with previous single-molecule techniques wherein only a subset of events can be detected in a specifically designed assay. Furthermore, unlike previous single-molecule techniques, HS-AFM allows us to know how individual molecules are really situated under assay conditions because of the visibility of both molecules and their local environments. Owing to these excellent features of HS-AFM imaging, the observed molecular events can be straightforwardly interpreted, leading to definitive conclusions; hypotheses and data interpretations play a much smaller role in deriving conclusions.

The high-resolution power of HS-AFM, capable of distinguishing individual domains and subunits within a protein molecule, is important, especially in identifying a local portion undergoing structural changes and finding interplay between subunits. For example, in the study of the $\alpha_3\beta_3$ subcomplex of F_1-ATPase (*i.e.*, rotor-less F_1-ATPase) in the presence of ATP [18], the individual subunits were clearly distinguished. Therefore, conformational changes occurring in a defined rotary sequence among the subunits were observed (Figure 2d), which led to a discovery that the intrinsic cooperativity responsible

Figure 2



HS-AFM images showing various dynamic events of proteins. **(a)** Wiggling motion of and order–disorder transitions observed in an intrinsically disordered region of facilitates chromatin transcription (FACT) protein. A shorter ID region belonging to the SPT16 subunit is deleted. Imaging rate, ~15 fps; scan area, 100 nm × 100 nm. **(b and c)** Stepping behavior (b) and foot stomp (brief detachment from and re-binding to actin) at the leading head (c) of myosin V. The arrow in (c) indicates detachment of the leading head from actin. Imaging rate, ~7 fps; scan area, 150 nm × 75 nm. **(d)** Counterclockwise rotary propagation of conformational changes of the $\alpha_3\beta_3$ subcomplex of F_1-ATPase in an ATP-containing solution (each 15 nm × 15 nm image was clipped from an 80 nm × 80 nm area image). The red dots indicate the highest pixel positions. Frame rate, 12.5 fps. **(e)** Light-induced conformational changes of D96N bR in the purple membrane. The illumination of green light was switched on and off at the beginning and the end of the second image, respectively. The white and light-blue triangles indicate a trimer and a trefoil, respectively. Imaging rate, 1 fps; scan area, 25 nm × 25 nm.

for torque generation to rotate the γ subunit is elicited through the β–β interplay alone, while the γ subunit is passively subjected to the torque. The high-resolution power also allowed the detection of small outwards movement (~0.8 nm) of the E–F helix loop of each bR molecule from the trimer center under light illumination (Figure 2e) [15]. The outwards movement results in the contact between three bR molecules (a triad designated as 'trefoil'), each belonging to an adjacent trimer (second frame in Figure 2e). This observation led to the discovery of positive and negative cooperativity effects of the bR–bR contact within a trefoil (not within a trimer) on the decay kinetics of the photo-activated bR; the kinetics is decelerated for a molecule early activated within a trefoil, whereas accelerated for a molecule activated last within a trefoil. It is hard to imagine how other approaches would allow the finding of these interplays between subunits or neighboring molecules which underlie the function or functional modulation of these proteins. As such, HS-AFM imaging will disclose many intriguing phenomena occurring in proteins, which other approaches may fail to notice, even for protein systems that have previously been scrutinized.

Future challenges in HS-AFM imaging

One of the greatest challenges in HS-AFM imaging is to observe the dynamic action of individual protein molecules on the surfaces of live cells and isolated organelles. This is because the surfaces are very soft and deformed by the contact with the AFM tip. For the relatively rigid surfaces of live bacterial and specialized eukaryotic cells, HS-AFM can visualize individual protein molecules and

their dynamics, as demonstrated in the imaging of densely packed porin (Msp1) molecules diffusing on the outer surface of the magnetotactic spirillum *Magnetospirillum magneticum* AMB-1 [11•] and of water channel aquaporin-0 binding to and dissociating from junctional microdomains on the lens cell surface [12•]. The surface rigidity depends on its size. Therefore, it will probably become possible to observe the dynamic molecular processes occurring on the surfaces of, for example, neuronal synapses and relatively small mitochondria and Golgi apparatuses (0.2–0.5 μm).

The direct visualization of morphological changes occurring in live cells is also a challenge in HS-AFM, because a large area has to be scanned at a high velocity. The recent progress of high-speed/wide-area scanners [13,41] and active vibration damping techniques [13] has made this visualization possible, as demonstrated in the visualization of various dynamic events occurring in live bacterial and eukaryotic cells; bacteriolysis caused by lysozyme [13] and an antimicrobial peptide [42], the process of endocytosis from the initial pit formation to budding of protrusions around the pits and their disappearance [13], and actin retrograde flow [13]. The resolution is higher than optical microscopy but the information acquirable with the HS-AFM imaging is limited and therefore cannot provide significant findings. To expand the usefulness, HS-AFM should be combined with fluorescence microscopy to make it possible to correlate the morphological changes with underlying molecular processes that proceed in the cell interior.

For this combination, HS-AFM needs to employ the tip-scan mode rather than the sample-stage scan mode. Very recently, a tip-scan HS-AFM system has been developed, allowing simultaneous capture of topographic and total internal reflection fluorescence microscopy images, as demonstrated [43••]. Various optical techniques can be implemented with this new HS-AFM system. For example, combining the system with the optical tweezers will allow the visualization of a single protein molecule under external force. Combining with tip-enhanced fluorescence microscopy [44,45] will materialize high-speed superresolution fluorescence microscopy, which allows simultaneous dynamic recording of high-resolution topographic and fluorescence images in the same field of view. Thus, HS-AFM technology has more potential to evolve and hence will become an even more useful tool in the biological sciences.

Conclusion

HS-AFM has recently opened new opportunities to directly observe biological molecules in action at high resolution, which allows more comprehensive understanding of how they function, even for those previously scrutinized, as demonstrated in the recent studies reviewed here. Because of this capability, HS-AFM will become more common and indispensable in biological sciences in the near future. The HS-AFM technology is well established, but is still evolving toward its use for a wider range of biological samples and phenomena. It is hard to imagine what will become possible once all of the technological capabilities of HS-AFM imaging have been fully realized.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Joo H, Balci H, Ishitsuka Y, Buranachai C, Ha T: **Advances in single-molecule fluorescence methods for molecular biology**. *Annu Rev Biochem* 2008, **77**:51-76.

2. Moerner WE: **New directions in single-molecule imaging and analysis**. *Proc Natl Acad Sci U S A* 2007, **104**:12596-12601.

3. Neuman KC, Block SM: **Optical trapping**. *Rev Sci Instrum* 2004, **75**:2787-2809.

4. Huang B, Bates M, Zhuang X: **Super-resolution fluorescence microscopy**. *Annu Rev Biochem* 2009, **78**:993-1016.

5. Vianni MB, Pietrasanta LI, Thompson JB, Chand A, Gebeshuber IC, Kindt JH, Richter M, Hansma HG, Hansma PK: **Probing protein–protein interactions in real time**. *Nat Struct Biol Mol Biol* 2000, **7**:644-647.

6. Ando T, Kodera N, Takai E, Maruyama D, Saito K, Toda A: **A high-speed atomic force microscope for studying biological macromolecules**. *Proc Natl Acad Sci U S A* 2001, **98**: 12468-12472.

7. Ando T, Uchihashi T, Fukuma T: **High-speed atomic force microscopy for nano-visualization of dynamic biomolecular processes**. *Prog Surf Sci* 2008, **83**:337-437.

8. • Ando T, Uchihashi T, Kodera N: **High-speed AFM and applications to biomolecular systems**. *Annu Rev Biophys* 2013, **42**:393-414.
Theoretical considerations of the highest possible imaging rate of AFM are described, together with some experimental designs required for HS-AFM imaging of proteins.

9. Ando T: **High-speed atomic force microscopy coming of age**. *Nanotechnology* 2012, **23**:062001.

10. •• Ando T, Uchihashi T, Scheuring S: **Filming biomolecular processes by high-speed atomic force microscopy**. *Chem Rev* 2014, **114**:3120-3188.
HS-AFM imaging studies of proteins are comprehensively reviewed. Especially, results obtained in the HS-AFM study of walking myosin V are described in detail, which is followed by detailed discussions of the chemo-mechanical coupling mechanism derived from this study and related unsolved questions to be addressed in the future.

11. • Yamashita H, Taoka A, Uchihashi T, Asano T, Ando T, Fukumori Y: **Single molecule imaging on living bacterial cell surface by high-speed AFM**. *J Mol Biol* 2012, **422**:300-309.
Dynamics of protein molecules on a live bacterial cell surface was visualized for the first time by HS-AFM.

12. • Colom A, Casuso I, Rico F, Scheuring S: **A hybrid high-speed atomic force–optical microscope for visualizing single membrane proteins on eukaryotic cells**. *Nat Commun* 2013, **4**:2155.
Dynamics of protein molecules on the surface of live eukaryotic cells was visualized for the first time by HS-AFM.

13. Watanabe H, Uchihashi T, Kobashi T, Shibata M, Nishiyama J, Yasuda R, Ando T: **Wide-area scanner for high-speed atomic force microscopy**. *Rev Sci Instrum* 2013, **84**:053702.

14. • Uchihashi T, Kodera N, Ando T: **Guide to video recording of structure dynamics and dynamic processes of proteins by high-speed atomic force microscopy**. *Nat Protoc* 2012, **7**: 1193-1206.
Experimental procedures and pre-cautions for HS-AFM imaging are described in step-by-step.

15. Shibata M, Uchihashi T, Yamashita H, Kandori H, Ando T: **Structural changes in bacteriorhodopsin in response to alternate illumination observed by high-speed atomic force microscopy**. *Angew Chem Int Ed* 2011, **50**:4410-4413.

16. Shibata M, Yamashita H, Uchihashi T, Kandori H, Ando T: **High-speed atomic force microscopy shows dynamic molecular processes in photo-activated bacteriorhodopsin**. *Nat Nanotechnol* 2010, **5**:208-212.

17. Yamashita H, Inoue K, Shibata M, Uchihashi T, Sasaki J, Kandori H, Ando T: **Role of trimer–trimer interaction of bacteriorhodopsin studied by optical spectroscopy and high-speed atomic force microscopy**. *J Struct Biol* 2013, **184**:2-11.

18. Uchihashi T, Iino R, Ando T, Noji H: **High-speed atomic force microscopy reveals rotary catalysis of rotorless F_1-ATPase**. *Science* 2011, **333**:755-758.

19. Shinozaki Y, Sumitomo K, Tsuda M, Koizumi S, Inoue K, Torimitsu K: **Direct observation of ATP-induced conformational changes of single $P2X_4$ receptor/channel**. *PLoS Biol* 2009, **7**:e1000103.

20. Yokokawa M, Takeyasu K: **Motion of the Ca^{2+}-pump captured**. *FEBS J* 2011, **278**:3025-3031.

21. Suzuki Y, Goetze TA, Stroebel D, Balasuriya D, Yoshimura SH, Henderson RM, Paoletti P, Takeyasu K, Edwardson JM: **Visualization of structural changes accompanying activation of N-methyl-D-aspartate (NMDA) receptors using fast-scan atomic force microscopy imaging**. *J Biol Chem* 2013, **288**:778-784.

22. Yokokawa M, Carnally SM, Henderson RM, Takeyasu K, Edwardson JM: **Acid-sensing ion channel (ASIC) 1a undergoes a height transition in response to acidification**. *FEBS Lett* 2010, **584**:3107-3110.

23. Sugimoto S, Yamanaka K, Nishikori S, Miyagi A, Ando T, Ogura T: **AAA+ chaperone ClpX regurates dynamics of prokaryotic cytoskeletal protein FtsZ**. *J Biol Chem* 2010, **285**:6648-6657.

24. Noi K, Yamamoto D, Nishikori S, Arita-Morioka K, Ando T, Ogura T: **High-speed atomic force microscopic observation of ATP-dependent rotation of the AAA+ chaperone p97**. *Structure* 2013, **21**:1992-2002.

25. Kodera N, Yamamoto D, Ishikawa R, Ando T: **Video imaging of walking myosin V by high-speed atomic force microscopy**. *Nature* 2010, **468**:72-76.

26. Colom A, Casuso I, Boudier T, Scheuring S: **High-speed atomic force microscopy: cooperative adhesion and dynamic equilibrium of junctional microdomain membrane proteins**. *J Mol Biol* 2012, **423**:249-256.

27. Casuso I, Khao J, Chami M, Paul-Gilloteaux P, Husain M, Duneau J-P, Stahlberg H, Sturgis JN, Scheuring S: **Characterization of the motion of membrane proteins using high-speed atomic force microscopy**. *Nat Nanotechnol* 2012, **7**:525-529.

28. Yamamoto D, Uchihashi T, Kodera N, Ando T: **Anisotropic diffusion of point defects in two-dimensional crystal of streptavidin observed by high-speed atomic force microscopy**. *Nanotechnology* 2008, **19**:384009.

29. Casuso I, Sens P, Rico F, Scheuring S: **Experimental evidence for membrane-mediated protein–protein interaction**. *Biophys J* 2010, **99**:L47-L49.

30. Yamashita H, Voïtchovsky K, Uchihashi T, Antoranz Contera S, Ryan JF, Ando T: **Dynamics of bacteriorhodopsin 2D crystal observed by high-speed atomic force microscopy**. *J Struct Biol* 2009, **167**:153-158.

31. Milhiet P-E, Yamamoto D, Berthoumieu O, Dosset P, Le Grimellec C, Verdier J-M, Marchal S, Ando T: **Deciphering the structure, growth and assembly of amyloid-like fibrils using high-speed atomic force microscopy**. *PLoS ONE* 2010, **5**:e13240.

32. Yilmaz N, Yamada T, Greimel P, Uchihashi T, Ando T, Kobayashi T: **Real-time visualization of assembling of a sphingomyelin-specific toxin**. *Biophys J* 2013, **105**:1397-1405.

33. Miyagi A, Tsunaka Y, Uchihashi T, Mayanagi K, Hirose S, Morikawa K, Ando T: **Visualization of intrinsically disordered regions of proteins by high-speed atomic force microscopy**. *Chem Phys Chem* 2008, **9**:1859-1866.

34. Hashimoto M, Kodera N, Tsunaka Y, Oda M, Tanimoto M, Ando T, Morikawa K, Tate S: **Phosphorylation-coupled intramolecular dynamics of unstructured regions in chromatin remodeler FACT**. *Biophys J* 2013, **104**:2222-2234.

35. Suzuki A, Hori T, Nishino T, Usukura J, Miyagi A, Morikawa K, Fukagawa T: **Spindle microtubules generate tension-dependent changes in the distribution of inner kinetochore proteins**. *J Cell Biol* 2011, **193**:125-140.

36. Igarashi K, Uchihashi T, Koivula A, Wada M, Kimura S, Okamoto T, Penttilä M, Ando T, Samejima M: **Traffic jams reduce hydrolytic efficiency of cellulase on cellulose surface**. *Science* 2011, **333**:1279-1282.

37. Shlyakhtenko LS, Lushnikov AY, Miyagi A, Lyubchenko YL: **Specificity of binding of single-stranded DNA-binding protein to its target**. *Biochemistry* 2012, **51**:1500-1509.

38. Suzuki Y, Gilmore JL, Yoshimura SH, Henderson RM, Lyubchenko YL, Takeyasu K: **Visual analysis of concerted cleavage by type IIF restriction enzyme SfiI in subsecond time region**. *Biophys J* 2011, **101**:2992-2998.

39. Wickham SFJ, Endo M, Katsuda Y, Hidaka K, Bath J, Sugiyama H, Turberfield AJ: **Direct observation of stepwise movement of a synthetic molecular transporter**. *Nat Nanotechnol* 2011, **6**: 166-169.

40. Miyagi A, Ando T, Lyubchenko YL: **Dynamics of nucleosomes assessed with time-lapse high-speed atomic force microscopy**. *Biochemistry* 2011, **50**:7901-7908.

41. Braunsmann C, Schäffer TE: **High-speed atomic force microscopy for large scan sizes using small cantilevers**. *Nanotechnology* 2010, **21**:225705.

42. Fantner GE, Barbero RJ, Gray DS, Belcher AM: **Kinetics of antimicrobial peptide activity measured on individual bacterial cells using high-speed atomic force microscopy**. *Nat Nanotechnol* 2010, **5**:280-285.

43. •• Fukuda S, Uchihashi T, Iino R, Okazaki Y, Yoshida M, Igarashi K, Ando T: **High-speed atomic force microscope combined with single-molecule fluorescence microscope**. *Rev Sci Instrum* 2013, **84**:073706.
Simultaneous capture of internal reflection fluorescence microscopy and HS-AFM images was materialized by the development of the tip-scan HS-AFM instrument, opening a new avenue to the evolution of HS-AFM technology.

44. Gerton JM, Wade LA, Lessard GA, Ma Z, Quake SR: **Tip-enhanced fluorescence microscopy at 10 nanometer resolution**. *Phys Rev Lett* 2004, **93**:180801.

45. Hayazawa N, Inouye Y, Kawata S: **Evanescent field excitation and measurement of dye fluorescence in a metallic probe near-field scanning optical microscope**. *J Microsc* 1999, **194**:472-476.