High-speed atomic force microscopy imaging of live mammalian cells

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Direct imaging of morphological dynamics of live mammalian cells with nanometer resolution under physiological conditions is highly expected, but yet challenging. High-speed atomic force microscopy (HS-AFM) is a unique technique for capturing biomolecules at work under near physiological conditions. However, application of HS-AFM for imaging of live mammalian cells was hard to be accomplished because of collision between a huge mammalian cell and a cantilever during AFM scanning. Here, we review our recent improvements of HS-AFM for imaging of activities of live mammalian cells without significant damage to the cell. The improvement of an extremely long (~3 μm) AFM tip attached to a cantilever enables us to reduce severe damage to soft mammalian cells. In addition, a combination of HS-AFM with simple fluorescence microscopy allows us to quickly locate the cell in the AFM scanning area. After these improvements, we demonstrate that developed HS-AFM for live mammalian cells is possible to image morphogenesis of filopodia, membrane ruffles, pits open-close formations, and endocytosis in COS-7, HeLa cells as well as hippocampal neurons.

Key words: Bio-imaging, live-cell imaging, nanotechnology, Probe microscopy, AFM

The possibility of direct visualization of live mammalian cells with high spatiotemporal resolution would provide the advanced knowledge of cellular functions. For example, morphology of synapses of neurons changes dynamically in response to extracellular stimulus and these morphological changes are critical for plasticity and adaptive response of neurons. However, since the size of synapses is only a few hundred nanometers, in order to better understand of the detailed processes of the morphological dynamics of synapses, direct visualization with nanometer resolution under near physiological conditions has long been desired, but remains a challenge.

Atomic force microscopy (AFM) can image a surface topography of objects with nanometer resolution in an aqueous solution and has been used to image a wide variety of morphological details of biological samples.
biological samples [1–11]. In particular, the appearance of high-speed AFM (HS-AFM), which has been extensively optimized the scanning speed by two orders of magnitudes faster than that of conventional AFM, opened a way to image the conformational change of single molecules on substrates with a subseconds time resolution [12,13]. In the past decade, various dynamic processes of biological samples including photo-induced conformational change of bacteriorhodopsin [14–16], myosin V walking on an actin filament [17], stabilization of membranes by annexin V [19], lipid membrane remodeling by ESCR-TIII polymerization [20], reaction processes of DNA targeting enzymes [21], nucleosome dynamics [22,23], local conformational changes of DNA strands [24–27], and dynamics of the nuclear pore complex [28,29], were visualized using HS-AFM. However, applications for nanostructure imaging of live mammalian cells has been complicated, since the length of scale of cells is three orders of magnitude larger than that of proteins. In this review, we introduce three improvements from the original HS-AFM setup to HS-AFM for imaging of live mammalian cells, and demonstrate HS-AFM movies of living COS-7 cells, HeLa cells and cultured hippocampal neurons by 5–10 μm fields in the time resolution of 5–10 seconds per frame, which visualized their cellular activities with nanometer resolution [30].

Development of HS-AFM for imaging of live mammalian cells

To apply HS-AFM for live-cell imaging, three improvements were required. First, we applied a wide-area scanner (photograph is shown in Fig. 1A), which is able to scan about 46×46 μm² within 50 seconds per frames [31]. In the mechanical design of a wide-area scanner, we referred the third-class leverage mechanism to amplify the displacements of X- and Y-directions. Specifically, both of the X- and Y-piezoelectric actuators (a nominal unloaded displacement is ~11 μm at 100 V) was symmetrically arranged against the supporting base attached a Z-piezoelectric actuator (a nominal unloaded displacement is ~4 μm at 100 V). In this case, the overall lever length is 25 mm. The fulcrum is set at the one end of the lever, while the force point is attached as a position of X- or Y-piezoelectric actuator with a length of 5 mm from the fulcrum; i.e., the designed lever ratio is 5. The actual displacements of this designed scanner resulted in ~46.7 μm and ~45.7 μm for the X- and Y-direction, respectively. A sample stage was glued on the top of a Z-piezoelectric actuator (white arrow in Fig. 1A). In addition, we further improved a wide-area scanner to obtain the best performance for HS-AFM observations, for example, a vibration damping for X-scan, a compensation for nonlinearity, a compensation for interference between X- and Y-scanners and the Z-scanner (Please see the details in our recent review [32]). Using this wide-area scanner, we can image a sample at ~7 second per frame for a scan area of 40×40 μm² at 256 pixels². The rate-limiting factor for imaging of the cell using a wide-area scanner is determined by the resonant frequency of the Z-piezoelectric actuator (f₀: ~50 kHz). Thus, the X-Y scanning size is enough to image a whole live mammalian cells. Indeed, we demonstrated HS-AFM movies of thin plasma membrane of live HeLa cells in 5×5 μm² with temporal resolution of five seconds per frame [31].

Second, a longer AFM tip was applied to avoid collisions between the base of cantilever and living cells. The small cantilever for HS-AFM consists of a bird-beak end with ~1 μm length (Left panel in Fig. 1B). We always fabricated an additional sharp tip on a bird-beak to obtain high-resolution images using electron beam deposition (EBD) by scanning electron microscopy (SEM) [33]. The length of an additional EBD tip used for imaging of biomolecules is less than ~1 μm. However, the tip of this length is unsuited for imaging of live mammalian cells, because the base of cantilever would collide with a taller region of a mammalian cell. To avoid these collisions, we fabricated a longer AFM tip by repeating 1 min EBD for 5–7 times on a bird-beak (the growth rate of an additional EBD tip is ~600 nm per min) [30]. To compensate the mechanical drift of the SEM, the focus position was reset after each EBD cycle. According to

**Figure 1** HS-AFM setup for observations of live mammalian cells. (A) Photograph of a wide-area scanner (B) Scanning electron microscopy (SEM) imaging of the end of the cantilever with and without an electron-beam-deposit (EBD) tip. The tip length is about 3 μm for live-cell HS-AFM. (C) Epi-fluorescence images of a COS-7 cell transfected with mEFP. The white broken lines highlighted the base of a cantilever. The white square indicates a HS-AFM scanning area. HS-AFM images corresponds to Figure 2.
mammalian cells without significant damages during HS-AFM scanning.

**Morphological dynamics of live COS-7 and HeLa cells at the leading-edge**

Using developed HS-AFM system, we first observed live-cell lines such as COS-7 and HeLa cells. Figure 1C shows the fluorescence image of a mEGFP transfected COS-7 cell. And the corresponding to a sequence of HS-AFM topographical images is shown in Figure 2. At the leading edge of a COS-7 cell, the HS-AFM movie shows constant membrane ruffling and extension or retraction of filopodium for at least 15 min (Fig. 2). We tried to confirm whether these cellular morphologies really relate to cell activities, we applied cytochalasin D, which inhibits actin polymerization [34]. As we expected, after the addition of cytochalasin D, those morphological dynamics at the leading edge observed before gradually abolished (Fig. 2). Subsequently, after washout for ~30 min by the imaging solution, morphological dynamics of COS-7 cells completely recovered, suggesting that membrane dynamics observed by HS-AFM requires actin polymerization.

Interestingly, the addition of some growth factors activates membrane dynamics at the leading edge of cells. After the addition of insulin, which is a hormone as a growth factor of mammalian cells, membrane ruffling became dramatically larger (dashed circles in Fig. 3A). Specifically, a height of the leading edge became taller and the speed of the repeated membrane ruffling was accelerated. Furthermore, some pits appeared on the cell surface, implying that endocytic events frequently occurred by the stimulation of insulin.

![Figure 2](image)

**Figure 2** HS-AFM images of a living COS-7 cell. A HS-AFM topographical image acquired from the area indicated in white box in Figure 1C before the addition of cytochalasin D (Top), after application of 20 ng/mL cytochalasin D (middle) and following washout for 30 min (bottom). HS-AFM images taken at the indicated times (green) and the image taken at 0 s (magenta) are overlaid. White arrows indicate newly appeared structures at the leading edge. HS-AFM imaging rates, 10 second per frame. HS-AFM pixel resolutions, 200×200 pixels².
Endocytosis of live COS-7 cells on the cell surface

We next observed the membrane dynamics at the center of live mammalian cells, where is closed to the nucleus (Fig. 4A). At the center of COS-7 cells, there is no unidirectional flow on the membrane surface. Instead, HS-AFM movie shows growths of protrusions in a vertical direction on the membrane surface and the appearance of the soft structure from the inside of the cell. In addition, many pits were observed on the membrane surface, and they constantly repeated the open and close forms on a specific area. In Figure 4B shows that time courses of the depth and height of the pits (Fig. 4B). To confirm pits formations are related to the cell activity, we applied a pharmacological experiment during HS-AFM observations. After the application of dynasore, which is an inhibitor of dynamin, pits formations were disappeared (Fig. 4C). Subsequently, formations of pits were recovered by washing out the drug for ~30 min by the imaging solution, suggesting that the observed pits on the cell surface are related to dynamin dependent endocytosis (Fig. 4C). When we overexpressed the constitutive active mutant of Rab5 (Rab5(Q79L)), which positively regulates endocytosis, HS-AFM movies of a COS-7 cells transfected with Rab5 mutant clearly shows that the frequency of pits formation increased, and the lifetime of pit was shorter than that of a COS-7 cell transfected with mEGFP (Fig. 4D, E). Thus, those results suggest that the observed pits on the cell surface are tightly associated with endocytosis. Interestingly, when pits closed, we often observed “cap-type” endocytosis, in which pits are closed with protrusions formed near the pits (Fig. 4B and Fig. 5). The height analysis in Figure 4B clearly shows the formation of the protrusion just after a pit closed (Fig. 4B). We hypothesize that a biological meaning of “cap-type” endocytosis is to gain the efficiency of a nutrient ingestion by once endocytosis, as if a protrusion cap is a scoop net. We noted that this “cap-type” endocytosis was also observed in COS-7, HeLa cells, as well as hippocampal neurons, implying the common endocytosis mechanism of mammalian cells (Fig. 8C). Moreover, pits repeatedly appeared on the specific area implying the existence of an endocytic “hot spot” on the cell surface (Fig. 4E).

Morphological dynamics of live neurons

From the above, the developed HS-AFM imaging of live mammalian cells could be performed for more than 30 min without any obvious damage to the cell. Using this HS-AFM, we next applied to directly visualize morphological dynam-
dissociated-cell cultures of hippocampal neurons from rats co-cultured with glia cells was the most suitable methods for HS-AFM observations. Figure 6 shows the Scanning Electron Microscopy (SEM) imaging of cultured hippocampal neurons of living neurons. On the other hand, we first required to optimize a culture method of neurons. After a process of many trials and errors for improving culturing methods for hippocampal neurons [35–38], we found that low-density dissociated-cell cultures of hippocampal neurons from rats co-cultured with glia cells was the most suitable methods for HS-AFM observations. Figure 6 shows the Scanning Electron Microscopy (SEM) imaging of cultured hippocampal...
structure belonged to the dendrite. Interestingly, this thin structure shows a quite flexible and changes its morphology over a few minutes (Fig. 9B). The rigid structure is 1500–2000 nm wide and ~590 nm high, while the thin flexible structure is ~140 nm high with different shapes. We note that those kinds of dynamics of the growth of filopodia at 9 DIV, the dendritic membrane ruffling at 13 DIV and the dynamics of small protrusion at 15 DIV were observed only living neurons on glia cells. This fact strongly suggests the importance of physical contacts between neurons and glia cells for their dynamic morphological changes.

Conclusions

The optimization of HS-AFM for live-cell imaging provides direct visualizations of morphological dynamics of live mammalian cells. Especially, the success of HS-AFM observations of living neurons would make possible to directly visualize the morphology dynamics of both pre- and postsynapses during their functions in the near future. After further improvements of HS-AFM techniques, such as combined with fluorescence resonance energy transfer (FRET) imaging or optical nanoscopy techniques [37–40], could add further information about conformational changes of specific receptors by external stimuli on membranes in living neurons.
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Notations

Please see HS-AFM movies of live mammalian cells in the reference No. 30.
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Conflicts of Interest

All the authors declare that they have no conflicts of interest.

Author Contribution

M. S. wrote the manuscript. H. W., T. U., T. A and R. Y. reviewed the manuscript and approved the final form.

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