Fast-scanning Atomic Force Microscopy

Combined AFM - Inverted Optical Microscope for Wet Cell Imaging

We describe herein the development of a fast-scanning atomic force microscopy (AFM) combined with inverted fluorescent microscopy (FM), aiming at the elucidation of dynamic structure-function relationships of biological macromolecules on live cell membrane. Applicability of the system is demonstrated by the correlated AFM-FM imaging of chicken red blood cell (RBC) surfaces.

Atomic force microscopies (AFMs) have been widely used in materials science and nanotechnology fields to observe and inspect nano-meter-sized structures. They have also become indispensable tools in the biological research community. The range of operation of the AFMs is well suited for characterizing structures at the molecular to cellular scales. Furthermore, the ability to operate in aqueous environment, without the need for sample treatment has made the AFM ideal for use in investigating biological samples such as proteins, nucleic acids, and even living cells/tissues, under physiological conditions. This remarkable advantage has made it possible for the dynamic processes of enzymatic reactions [1-4] and morphological changes of living cells [5-8] to be studied. Finally, Ando's successful development of fast-scanning AFM has made a breakthrough in the challenge to the slow data acquisition rate of earlier models of conventional AFM (several seconds to minutes per frame) towards a much faster rate of >1 frame per second (fps) [9]. Various applications of this device have quickly appeared in the last decade. Conformational changes of proteins [10-13], reaction mechanisms of DNA targeting enzymes [14-17], and dynamic behavior of motor proteins [18] and nucleosomes [19, 20] have been addressed by the fast-scanning AFM. It is noteworthy that, presently, both the 'sub-second time frame' and 'nano-meter scale' single molecule observations of functional biological macromolecules cannot be achieved by other techniques.

Our long-standing interest has been in the real-time high resolution imaging of 'living cell' surface. Thus, we have built a novel tip-scan type of high-speed AFM unit which can be set up on the sample stage of an inverted optical microscope.
This instrument has enabled us to monitor the location of the AFM cantilever over the cell surface and to create correlated optical and fast-scanning AFM datasets.

Here, we report the basic instrumentation of this newly constructed device and its application to the imaging of membrane surfaces.

**Description of Imaging System**

A tip-scan, high-speed AFM unit is designed to be set up on the sample stage of an inverted Optical Microscope (Olympus IX71) as shown at figure 1A. The external appearance of the AFM unit is shown in figure 1B and its size and weight are 285 × 166 × 112 mm$^3$ and 4.5 kg respectively. It is mandatory for the resonance frequency of the X-Y-Z scanner unit to be high enough, hence its size is designed to be compact. A sharp probe is also required to observe the details of the biological specimens. This is achieved by using a cantilever (width: 2 μm, length: 9 μm) which has a carbon nanofiber (CNF) probe (diameter: 14 nm, length: 80 nm) (Olympus; BL-AC10FS-A2) [21] shown in figure 2. The displacement of the cantilever is detected by the movement of the laser diode (LD) beam which is focused on the cantilever surface by an NA: 0.4 objective lens (the spot size is 2-4 μm) and reflected by the cantilever surface and goes to the photo detector (PD) (this is the so called optical lever method). The scan size of the X-Y-Z cantilever-scanner which is composed of layered piezoelectric bodies (PZTs) is 2 × 1.5 × 0.4 μm$^3$ (when applied voltage is 100V) and the resonance frequency of the X-Y-Z scanner is 150 kHz in the Z direction. Consequently, we get an AFM image within 0.1 second (at max speed).

The soft nature of biological samples also renders them highly susceptible to damage by the AFM probe in the course of image acquisition. We circumvent this problem by detecting, in high sensitivity, the force applied to the specimens by the probe. We have accomplished this by detecting the displacement of the cantilever and improving the sensitivity of the cantilever displacement sensor. These two procedures are achieved by detecting the change of the amplitude and phase of
vibration of the cantilever in high sensitivity as well as controlling the position of the cantilever precisely using the signal produced by the change. As a result, biological specimens are hardly damaged in the course of AFM imaging.

The optical microscope works independently from the AFM unit without any influence on scanning motion of the cantilever. Thus, correlated optical (fluorescent) and fast-scanning AFM images can be recorded.

**Demonstration of the System**

We tested our imaging system on lipid expansion on a mica surface. The mica disk (diameter 1.5 mm) was soaked in MgCl₂ (5 mM) solution, and 50μL of liposome solution (2.5mM) made of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS), and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2,1,3-benzoxadiazol-4-yl) (DOPE-NBD) (molar ratio, at 3: 1: 0.05) was added thereafter. An example of continuous observation (motion picture observation) is shown in figure 3. Figure 3A shows a fluorescence microscope image of mica, falling fluorescent liposome and cantilever for AFM imaging. At first, a liposome falls on mica substrate, then it ruptures by gravitational or electrostatic force, and finally the lipid bilayer expands on mica (fig. 3B). Figure 3C shows a series of AFM images which represent the spreading lipid bilayer on mica. The stages in the formation of the lipid bilayer of 4nm thickness are monitored by the motion picture of 0.5 fps together with fluorescent movies, demonstrating the capability of the system to obtain correlated fluorescent and fast-scanning AFM image sets.

**Biological Application**

As an initial step in biological application of the AFM, we opted to examine red blood cells (RBCs) because they contain a well-developed membrane skeleton network which is made of the lipid bilayer and the spectrin based cytoskeletal structure lining the cytoplasmic surface of the lipid membrane [22].

Figure 4 shows an example of the observation of chicken RBCs. Optical microscopy imaging revealed their characteristic oval shape (head diameter 7-10 μm) with nucleus (fig. 4A, B). The location of the cantilever over the cell membrane stained with 3-Hexyl-2-[3-(3-hexyl-2(3H) benzoxazolylidene)-1-propenyl] benzoxazolium iodide (DiOC6) was able to be determined by optical microscopic views. Thus, AFM image of the specific position of the surface can be gained and be overlaid with fluorescent images (fig. 4C). Importantly, AFM imaging of the RBCs surfaces revealed a meshwork structure, suggesting that the cytoskeleton network in living cells can be monitored at the extracellular surface of the plasma membrane (fig.
4D).

**Conclusion**

The tip-scan type of high-speed AFM we introduced here has opened a new phase of nano-scale analysis of living cell surfaces. Optimized feedback system of the AFM has allowed us to visualize the topography of soft biological specimens (such as artificial lipid membrane, live cell membranes and tissue surfaces) in physiologically relevant buffer conditions at the scanning rate of 0.1-10 fps. More importantly, by combining inverted optical microscopy with this AFM system, the position of the AFM cantilever over the sample surface can be monitored, and optical and fast-scanning AFM images of the targeted point can be correlated. Thus, the present system offers new ways of addressing the surface structure, molecular organization, conformational changes and dynamics of membrane-embedded proteins on living cell surface.

**References**

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