Topographical Imaging of Soft Structures of Lipid Membranes at Water–solid Interface by Fluorescence Interferometry

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We have developed a modified fluorescence interference contrast microscopy (FICM) that facilitates height measurement of soft structures of lipid membranes in nm-scale. The resolution of the method was examined by measuring depth of micropits on glass substrates covered with bilayer lipid membranes, and then thickness of giant membrane lobes was measured as a practical example of soft structures.

For past decades, lipid membranes of simple morphologies such as, supported bilayer lipid membranes (s-BLM) on solid surfaces and spherical vesicles (liposomes) dispersed in aqueous solutions have received much attention to establish the chemical and physical bases of biomembranes. Recently, the developments in microscope techniques for imaging, fabrication, and manipulation have accelerated the studies on lipid membranes of unconventional morphologies formed at water–solid interface such as adherent giant vesicles,[1] microscopic liposome networks,[2] ruptured giant unilamellar vesicles on s-BLM,[3] and giant membrane lobes[4] in order to explore further functionality of lipid membranes. Fluorescence microscopy is one of the best methods to reveal these soft structures of lipid membranes without mechanical perturbations, since fluorescence measurements are so sensitive that one can determine the number of lamellas based only on their fluorescence intensity. However, in general, conventional fluorescence microscopy dose not provide information on topography (local height, depth, or thickness) of objects. Recently, taking advantage of optical standing waves formed near the surface of oxidized silicon wafers under a fluorescence microscope, Fromhertz et al. have proposed fluorescence interference contrast microscopy (FICM) which provides contour mapping of the topography of living cells put directly on an oxidized silicon wafer.[5] However, for nm-scale topography down to 1/4 of wavelength of excitation light (λ_{ex}), no contour line was obtained in the FICM images and it is difficult to measure the topography without using the special oxidized silicon wafers with multiple-thickness oxide layers.[5–7]

In this letter, we propose a modified FICM that provides fluorescence images composed of sufficient number of contour lines even for nm-scale topography. The key point of our modification is in the use of Wiener's optical arrangement on which the substrate is set on a mirror at small inclination angle.[8] At first we examined the resolution of the modified FICM by measuring known depths of micropits on glass substrates covered with s-BLM, and then we have demonstrated the thickness measurement of giant membrane lobes.

The glass substrates with micropits prepared by HF etching, optically flat silica glass substrates, and n-type (111) silicon wafers were cleaned by immersion into a solution (NH_4OH:H_2O_2:H_2O = 1:1:10) at 80 °C for 15 min before use. In this process, the silicon wafers was oxidized to form thin oxide layer (<1 nm). Prior to use, the depth of the micropits was measured using an atomic force microscope (AFM) (Nanoscope IIIa, Digital Instruments). Chloroform solution of phospholipid and perylene (ca. 0.5 mol%) was prepared using 99 or 60% L-α phosphatidylcholine from egg yolk (Egg-PC) purchased from SIGMA. Using the chloroform solution, small unilammellar vesicles (SUV) were prepared roughly according to the Barenholz procedure.[9] s-BLM was formed on the substrates by fusion of the SUV onto the substrates,[10] while the giant membrane lobes were prepared on the substrates in a NaCl solution as follows. The chloroform solution of 2 μL was painted on a glass rod of diameter about 0.1 mm. By bringing the side of the glass rod in contact with the clean surface of the substrates, the dried lipid films were left on the glass rod, and they were stamped onto the substrates to deposit a line of phospholipid as a lipid source. Then, the substrate was set in a salt solution (10 mM Tris-HCl buffer, pH 8, 100 mM NaCl) at 25 °C to bring about a spreading phenomenon of membrane lobes along the substrates. Since each of the lipid bilayers was doped with perylene (i.e. a fluorescent probe which is trapped selectively in hydrophobic regions in bilayer lipid membranes), they were observed by an incident light fluorescence microscope (BX-50, Olympus) using the emission line at 404.7 nm of a high-pressure mercury lamp as an excitation light.

Figure 1a shows our experimental system for the depth measurement of the micropits to examine the resolution. As a mirror, an oxidized silicon wafer was put in the 100 mM NaCl solution under a fluorescence microscope. Near the surface of oxidized silicon wafer, standing waves of excitation light were formed.[5] On the oxidized silicon wafer, a glass substrate with micropits covered with s-BLM was set at small inclination angles ($\tan\theta$ = 0.0030–0.0075) to the oxidized silicon wafer. Accordingly, when perylene molecules in the s-BLM were strongly excited at the anti-node planes of the standing wave, the excited areas were observed as bright bands by fluorescence microscopy as shown in Figure 1b. The point **A** indicates a position of a

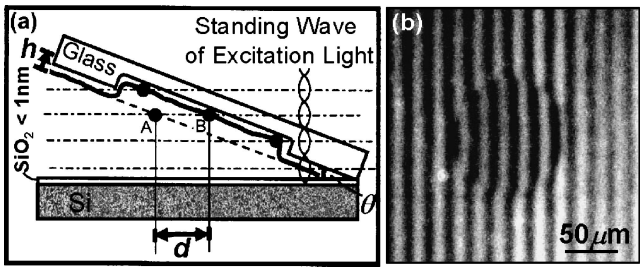

Figure 1. The depth measurement of micropits. (a) the schematic illustration of the side view of experimental system under fluorescence microscopy, the bold free-hand line indicates the s-BLM, (b) the modified FICM image of the s-BLM on a micropit of 72 nm in depth.

bright band (band **A**) at which an anti-node plane meets the s-BLM outside of the micropit. The point **B** indicates the position of the bright band (band **B**) at which the same anti-node plane meets the s-BLM inside of the micropit. Measuring the distance d between the bands **A** and **B** (i.e. a shift of the interference bands), the depth h of the micro-pit is given by $h = d \sin\theta$. For micro-pits of known depths of 8, 15, 33, 72, and 265 nm (measured by AFM), the value of h was 9, 16, 39, 74, and 271 nm, respectively. Therefore, we can conclude that our method has nm resolution.

Figure 2a shows a fluorescence image of giant membrane lobes used for the thickness measurement by the modified FICM. The lobes grew on a single lipid bilayer spreading from a lipid source on the silica glass substrate. We confirmed that the fluorescence intensity of the single lipid bilayer agrees with that of s-BLM. Since all the lobes were well adjusted in focus, the height of them was less than the focal depth (<0.5 μm). Figure 2b shows fluorescence intensity measured along the broken lines in Figure 2a. According to the fluorescence intensities, most of the lobes have fluorescence intensities correspond to two bilayers or four bilayers. Figure 2c shows a schematic illustration of the most feasible structure of the lobes composed of two bilayers. The growth of the lobes can be observed also on the oxidized silicon wafers, however, no contour line could be observed in normal FICM images of the lobes on the oxidized silicon wafer, indicating that the thickness of the lobes is smaller than 101 nm (i.e. 1/4 of λ_{ex}). Figure 3a shows our experimental system for the modified FICM of the lobes composed of two bilayers. The points **A**, **B**, and **C** correspond to the positions of bright bands (bands **A**, **B**, and **C**) at which an anti-node plane meets the single lipid bilayer, the lower lipid bilayer of the lobe, and the upper lipid bilayer of the lobe, respectively. Since the thickness of the lobes is smaller than the 1/4 of λ_{ex}, the band **B** and the band **C** almost overlap with each other and seem to be a single brighter band that is observed at the middle point between the points **B** and **C**. The point **M** corresponds to the position of the brighter band (band **M**). Measuring the distance d_M between the band **A** and the band **M**, the thickness h_M can be given by $h_M = d_M \sin\theta$, where the h_M corresponds to average height of

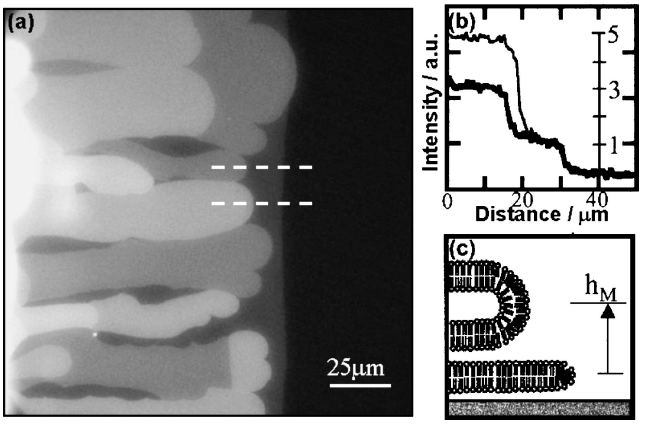

Figure 2. The lateral growth of membrane lobes on a silica glass substrate in 100 mM NaCl. (a) A fluorescence image of phospholipid membranes spreading from the left to the right, (b) bold and thin lines indicate the fluorescence intensities measured along the upper and lower broken lines in (a), respectively, (c) a schematic illustration of the front of membrane lobes composed of two bilayers growing on the single lipid bilayer.

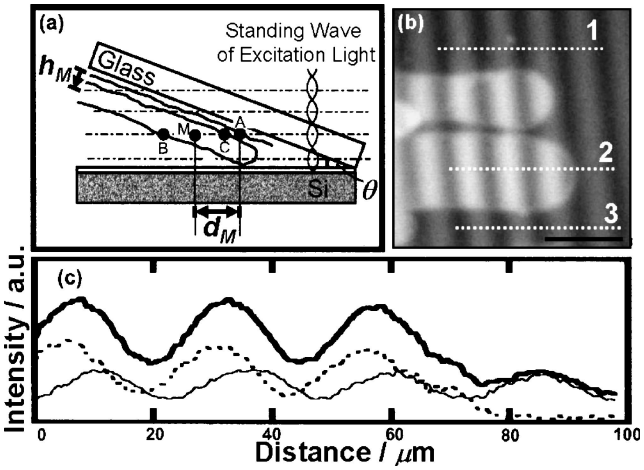

Figure 3. The thickness measurement of membrane lobes composed of two bilayers. (a) the schematic illustration of the side view of the experimental system under a fluorescence microscope, the bold free hand lines indicate the lipid bilayers, (b) the modified FICM image of lobes of 60% Egg-PC (bar = 50 μm), (c) the bold line indicates the fluorescence intensity measured along the line **2** in (b) and the thin line indicates the fluorescence intensity of the single lipid bilayer along the line **2** calculated based on the fluorescence intensities measured along the lines **1** and **3** in (b) and the dotted line indicates the fluorescence intensity of the membrane lobes given by the difference between the bold line and the thin line.

the two bilayers that form the lobe on the single lipid bilayer as shown in Figure 2c. Figure 3b shows an example of the modified FICM image of the lobes composed of two bilayers and Figure 3c shows spatial distributions of fluorescence intensities measured or calculated based on Figure 3b. The thin line indicates the fluorescence intensity of the single lipid bilayer along the line **2** in Figure 3b and each peak corresponds to the band **A** for each anti-node plane, while the dotted line indicates the fluorescence intensity of the lobes along the line **2** and each peak corresponds to the band **M** for each anti-node plane. Therefore, measuring the shift between the thin line and the dotted line, we could obtain the values of d_M. For 99% Egg-PC and 60% Egg-PC, the values of h_M were 18 and 38 nm, respectively. This means that our method is sensitive to nm-scale difference in topography reflecting the composition of lipids.

In conclusion, we could measure the topography of membrane lobes fixed at water–solid interface by the modified FICM. We believe that the method is a powerful tool to study a variety of soft structures of molecular aggregates, since the method is very simple and applicable for nanometer thick systems on a variety of transparent substrates.

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