Localized surface plasmon microscopy of submicron domain structures of mixed lipid bilayers

Koyo Watanabe,1 Ryosuke Miyazaki,2 Goro Terakado,2 Takashi Okazaki,3 Kenichi Morigaki,3,4 and Hiroshi Kano2,*

1Unit of Measurement Technology, CEMIS-OUUL, University of Oulu, PO Box 51, 87101 Kajaani, Finland
2Department of Electrical and Electronic Engineering, Muroran Institute of Technology, Mizumoto 27-1, Muroran, Hokkaido 050-8585, Japan
3Research Institute for Cell Engineering, National Institute of Advanced Industrial Science and Technology (AIST), Ikeda 563-8577, Japan
4Research Center for Environmental Genomics, Kobe University, Nada-ku, Kobe 657-8501, Japan

Abstract: We propose scanning localized surface plasmon microscopy of mixed lipid bilayers with submicron domain structures. Our observation technique, which employs localized surface plasmons excited on a flat metal surface as a sensing probe, provides non-label and non-contact imaging with the spatial resolution of ∼170 nm. We experimentally show that submicron domain structures of mixed lipid bilayers can be observed. A detailed analysis finds that the domains are classified into two groups.

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References and links

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1. Introduction

It is recognized that mixed bilayers are useful to model lateral heterogeneity of biological membranes as observed in lipid micro-domains. Among the model systems of mixed bilayers, substrate-supported planar lipid bilayers (SPBs) [1–3] have been receiving heightened attention due to their high potential to host a variety of bio-functions on a sensing surface of various bio-sensors. Since SPBs affect on behavior and functionalities of molecules supported in SPBs (e.g., SPBs with domain structure influence protein concentration, protein transport, mobility, and so on [4–6]), further understanding on SPBs is important. For the moment, one of the problems seen in the study of SPBs is brought by the morphological complexity which sometimes prevents clear observation of local bio-functions, so that, it is necessary to widen the selection of observational methods.

Criteria required in the observational method can be listed as follows; (a) spatial resolution to observe domain structures with the size of a sub-micrometer; (b) thickness resolution to be able to distinguish lipid bilayers with a precision of a few nanometers; (c) non-label measurements and measurement in an aqueous environment to maintain the lipid’s original nature; (d) no physical obstructions over the lipid bilayers that could prevent the flow of molecules and liquids fluidity.

In contrast to these criteria, even the atomic force microscopes (AFMs) that have a cantilever approaching to the sample surface are not perfect instruments because the cantilever could impede the liquid fluidity and aqueous environment leads to loss of sensitivity, while AFMs provide observation of lipid bilayers at molecular scale, and remarkable outcomes in this field [4, 7, 8]. Fluorescence microscopes are also imperfect instruments because fluorescent dyes that are usually required for observation affect the lipid molecule’s behavior in some cases [9], while fluorescence microscopes provide knowledge of lipid nature such as lipids distribution and diffusion speed.

Among the various instruments, a localized surface plasmon (LSP) microscope [10–12] satisfies all the criteria. In this microscope, surface plasmons excited in the microscopic region on the metal surface are used as a virtual sensing probe against refractive index on the metal surface. As the size of the sensing region is a few hundred nanometers [13, 14], imaging with high spatial resolution is achievable. The microscope has been experimentally demonstrated the imaging of the lipid bilayers with high thickness resolution by non-contact, non-label measurement [14]. Regarding other criteria, there is no obstruction to prevent the flow of molecules and liquids fluidity because a physical probe is not necessary. Fluorescent dyes are not necessary, as well.

In this paper, we report that domain structures of mixed lipid bilayers which have the size of
sub-micrometer can be observed by the scanning localized surface plasmon microscope for the first time.

2. Localized surface plasmon microscope

A surface plasmon, which is a quantum of collective electron oscillation on a metal surface, can be excited by light under certain optical configurations. Figure 1(a) shows a configuration widely used for exciting surface plasmons. It consists of a glass prism coated with a thin metal film [15]. In this configuration, the metal film is illuminated from the side of the glass prism. By using this configuration, a plane wave having an appropriate incident angle and polarization excites the surface plasmons on the surface of the metal facing the opposite side of the prism. The excited surface plasmons propagate on the metal surface as shown in Fig. 1(b).

![Fig. 1](image.png)

Fig. 1. (a) Conventional configuration for exciting surface plasmons. (b) Surface plasmon propagation on thin metal film and its propagating direction. (c) Configuration for exciting LSP. The two arrows indicate the optical path for the excitation of surface plasmons. (d) Surface plasmons propagating to the optical axis and their localization.

Figure 1(c) shows a setup, in which a focusing beam propagating in immersion-oil is employed to illuminate a thin metal film on a glass substrate. When the numerical aperture of the oil-immersion lens is large enough, plane wave components satisfying the excitation condition of the surface plasmon are included in the focusing beam. With this setup, surface plasmons propagating to many directions are excited, and interference between these surface plasmons provides localization in the microscopic region as shown in Fig. 1(d).

The surface plasmon is widely used as a sensitive measurement probe of the refractive index because the excitation angle of the surface plasmon shows high dependency on refractive index on the metal surface. In the case of the setup shown in Fig. 1(c), the excitation angle can be obtained from reflected light distribution. In the typical cases, a dark ring or arcs appears in the light distribution of the reflected light due to absorption by the surface plasmon excitation. The radius of curvature of the ring or arcs reflects the excitation angle, so that variation of the refractive index on the metal surface can be measured.
Figure 2 shows calculated results of the reflected light intensity distribution at the exit pupil. The distribution was obtained by using the multi-reflection theory described in Ref. [10] under the assumption of radial polarization for the illumination light. In this calculation, we assumed the wavelength of the light in the vacuum as 632.8 nm, the numerical aperture of the lens as 1.65, the thickness of the metal as 44 nm. We also assumed the refractive index of the metal film and a sample on the metal surface as 0.300 + 3.089i and 1.330, respectively. In the calculated distribution, one can observe a dark ring. Since the radius \( \rho_{sp} \) changes owing to the refractive index on the metal surface, we can detect the local effective refractive index \( n_s \). The relationship can be approximately expressed in the following equation:

\[
\rho_{sp} = n_g \frac{\omega}{c} \sin \theta_{sp} \simeq \text{Real} \left( \frac{\omega}{c} \left( \frac{n_m^2 n_s^2}{n_m^2 + n_s^2} \right)^{1/2} \right). \tag{1}
\]

where \( \omega, c, n_g, \theta_{sp} \) and \( n_m \) denote angular frequency, speed of light in vacuum, refractive index of glass, excitation angle of surface plasmon, and complex refractive index of the metal, respectively [16]. By using this relationship, we can determine the local effective refractive index on the metal surface. A refractive index distribution can be obtained by scanning the sample in two dimensions. This is the observation principle of the scanning LSP microscope.

Here, we briefly summarize the thickness resolution against a film sample produced on the metal surface [14]. To determine it, dispersion of the measured refractive index is firstly obtained by using an uniform medium with known refractive index. This dispersion expresses stability of the measurement. Secondly, effective refractive index of a lipid bilayer with known thickness is measured to find the dependency of thickness on effective refractive index. By combining these relationships, the thickness resolution of the microscope can be determined.

In the previous study on lipid bilayers, the thickness resolution of our LSP microscope was estimated as \(~0.33 \text{ nm}\) [14]. It has been also confirmed that LSP microscope can distinguish thickness difference between DLPC (1,2-Dilauroyl-sn-glycero-3-phosphocholine) bilayers with the thickness of 3.0 nm and DiynePC ((1,2-bis(10,12-tricosadiynoyl)-sn-glycero-3-phosphocholine)) bilayers with the thickness of 4.6 nm.

Spatial resolution of this microscope is determined by the electric field distribution of LSP, and it depends on the polarization of the excitation light. Since radial polarization minimizes the size of the electric field distribution, the highest spatial resolution is provided by this polarization [11, 13]. Figure 3(a) shows the electric field intensity distribution on the metal surface. We calculated the distribution by superposing plane wave components involved in the converging light after multiple reflections in the Kretschmann configuration [11]. We assumed radially...
polarized light with the wavelength of 632.8 nm focused by a objective lens with the numerical aperture of 1.65. We also assumed a Au film with the refractive index of 0.300 + 3.089i and the thickness of 44 nm covered by water with the refractive index of 1.330. Figure 3(b) shows the intensity plot corresponding to the white dotted line shown in Fig. 3(a). From the plot, one can see that the full width at half maximum (FWHM), which represents the spatial resolution of the microscope is $\sim 180$ nm.

Fig. 3. Calculated electric field intensity distribution of LSP assuming radially polarized light for excitation. Image (a) is the distribution on the Au surface facing pure water. The plot shown in Fig. 3(b) is the profile along the white dotted line on image (a).

In the case of lipid bilayer measurements, it is preferable to cover the metal surface with a SiO$_2$ layer to deposit the lipid bilayers. When we assume that the refractive index and thickness of the SiO$_2$ layer are 1.515 and 15 nm, respectively, and the SiO$_2$ faces water, the FWHM on the SiO$_2$ is increased to $\sim 170$ nm [14] because the wavenumber of surface plasmons increases due to higher effective refractive index on the metal surface.

Fig. 4. Apparatus of the LSP microscope.

Figure 4 shows the optical apparatus of the microscope. A He-Ne laser operated at the wavelength of 632.8 nm is used as a light source. The laser is expanded and collimated with lenses L1 and L2. Polarization of the expanded light is converted from linear to radial by a device which consists of a $\pi$-step phase plate and a liquid crystal cell [17, 18]. The radially polarized
beam is relayed onto the entrance pupil of an oil immersion objective lens by use of a Telecentric imaging system (L3 and L4) to suppress the diffraction from the polarization converting device. The relayed light is focused on the metallic thin film, and the reflection is collected by the same objective lens. The light intensity distribution at the exit pupil of the objective lens is imaged onto the CCD by a lens L5. The image recorded by the CCD is transferred to a PC, and processed to determine the local effective refractive index on the metal surface. To create a refractive index image, the sample substrate is scanned in two dimensions by using a piezo-stage. The substrate surface is covered with flow cell to keep the sample in aqueous solution. For the excitation of the surface plasmon on the metal surface covered with aqueous solution, the numerical aperture of the objective lens is chosen as 1.65.

3. Results and discussion

3.1. Observation of DSPC lipid bilayers

To study properties of mixed lipid bilayers, we chose DSPC (1,2-Distearoyl-sn-glycerol-3-phosphocholine) and DLPC because these two lipid separate into two phases, DSPC forming a solid (gel)-phase and DLPC forming a liquid-crystalline phase. The two domains have a clear thickness difference as has been previously observed by AFM [19]. Before examining the mixed lipid bilayers, we determined the effective refractive index of DSPC bilayer, while that of DLPC bilayer had been determined in our previous work [14].

![Schematic outline of bilayer patterning procedure and target area for observing lipid samples.](image)

Fig. 5. Schematic outline of bilayer patterning procedure and target area for observing lipid samples.

In order to obtain the effective refractive index of DSPC bilayer, we produced DSPC bilayers incorporated in micro-meter gaps of patterned lipid bilayers. Figure 5 shows a schematic outline to prepare the patterned bilayer. Figure 5(a) shows the lipid bilayer of monomeric DiynePC deposited on the solid substrate. The DiynePC lipids contain diacetylene moieties, and they are polymerized by UV irradiation. Figure 5(b) shows the irradiation process. The polymerized patterns were created by irradiating the bilayer through a quartz plate, where a patterned
chromium layer was coated as a mask. After UV irradiation, we removed the unpolymerized DiynePC molecules from the substrate with 0.1 M SDS solution at 30°C for 30 min, and then the solutions were thoroughly rinsed with deionized water. As shown in Fig. 5(c), we obtained the polymerized patterns frame. The lipid bilayers to be analyzed can be deposited in gaps of patterned lipid frame by the vesicle fusion method, as shown in Fig. 5(d). During the vesicle fusion process, the substrate deposited with the patterned lipid bilayers was heated above the phase translation temperature of DSPC lipids (55°C). Then, the substrate was cooled down to room temperature. Loosely deposited bilayers on the patterned lipid bilayer in this vesicle fusion process can be removed by rinsing with PBS solution. The substrate prepared by this process was observed by the LSP microscope. We subsequently introduced SDS solution to remove the DSPC lipids from the substrate and observed the same region after we replaced the SDS solution with deionized water to calibrate the measured value.

(a)

Effective refractive index

1.340

1.330

5 µm

(b)

Fig. 6. (a) Effective refractive index distribution of DSPC lipid bilayer in patterned lipid bilayers observed with the LSP microscope. DSPC lipid bilayers can be found as higher effective refractive index than that of patterned lipid bilayer. (b) Observed effective refractive index distribution after removing DSPC bilayers. The black circular region shows the gap of the patterned lipid bilayers.

Figure 6(a) shows observed results of DSPC bilayers in the patterned lipid bilayer, which
has lattice gaps with the size of 4 \( \mu m \). Figure 6(b) shows the image after removing the DSPC lipids. These images are observed in the region of 15 \( \mu m \times 15 \mu m \) with measurement points of 64 \( \times \) 64. From these images, the incorporated DSPC bilayers in the gaps can be clearly observed. The effective refractive index of the DSPC bilayers with one standard deviation is 1.3381\( \pm \)7.1 \( \times \) 10\(^{-4} \) [RIU\(_{\text{eff}}\)], which is calibrated by using deionized water having a refractive index as 1.330. The measured value is \( \sim \)2 \( \times \) 10\(^{-3} \) [RIU\(_{\text{eff}}\)] higher than the effective refractive index of patterned lipid bilayers. This property indicates that the DSPC produces thicker bilayers, and publications on the thickness of the patterned lipid bilayers (4.6 \( \text{nm} \) [20]) and DSPC bilayers (4.8 \( \text{nm} \) [4]) support this result.

With regard to the DLPC lipid bilayers with a thickness of 3.0 \( \text{nm} \) [21], the effective refractive index was measured as 1.3336\( \pm \)3.0 \( \times \) 10\(^{-4} \) with the LSP microscope [14].

### 3.2. Observed properties of a mixed lipid bilayer

In order to observe the mixed lipid bilayers, we prepared vesicles from a mixture of DLPC and DSPC with the ratio of 1:1. We deposited them into the gap of the patterned lipid bilayers by the vesicle fusion method. The size of the gap was 10 \( \mu m \) square. To create domains of lipid bilayers in the gap by using temperature controlling method [4], we heated the substrate to 80\(^\circ\)C during the vesicle fusion process. We cooled down the substrate to the room temperature and removed loosely adsorbed lipids on polymeric bilayers with the buffer solution.

![Fig. 7.](image-url) Fig. 7. (a) Effective refractive index distribution of mixed lipid bilayers in a 10 \( \mu m \) gap of patterned lipid bilayers observed with the LSP microscope. The white framed area shows the center part of a gap of patterned lipid bilayers, and domains due to the mixed lipid bilayer can be seen within it. (b) Histogram of effective refractive index in white framed area A.

Figure 7(a) shows the image of the effective refractive index distribution. The size of the observed region was 15 \( \mu m \times 15 \mu m \) with the pixels of 80 \( \times \) 80. In this image, a square corral with domains was observed. To evaluate the domains in detail, we produced a histogram of the effective refractive index from the framed area A. As shown in Fig. 7(b), the histogram revealed two peaks, so that we supposed that the domains were classified into two groups. From the comparison of these value with the effective refractive indices of homogenous DLPC and DSPC bilayers, we conclude that the gap consists of DSPC dominant domains and DLPC and DSPC mixed domain because the peak with larger effective refractive index and the other
peak had the value close to that of the homogeneous DSPC bilayer and the mean of the values of homogenous DLPC and DSPC bilayers, respectively.

4. Conclusions

We have demonstrated imaging capability of the LSP microscope by observing the submicron domain structure produced in the mixed lipid bilayer. In this observation, we introduced patterned lipid bilayers having lattice gaps with the size of 10 µm. The observed image of the mixed DLPC and DSPC bilayers deposited into the gaps showed the domain structure. Further analysis on the effective refractive index, we suppose that principal components of the domain can be found.

The LSP microscope has the potential to record domain formation processes by achieving a faster imaging speed.