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Label-free quantification of cell-to-substrate separation by surface plasmon resonance microscopy

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ABSTRACT

We study surface plasmon resonance microscopy (SPRM) for label-free quantification of cell-to-substrate separation. We have established a depth extraction model in which we compare a layered cell substrate model with resonance characteristics obtained by SPRM. We have applied the model to human aortic endothelial cell (HAEC) culture and determined the separation distance to be 40–60 nm. We have also investigated the precision of the SPRM model associated with the deviation in the model parameters, which is estimated to be 15 nm. The results can serve as the basis for more extensive cell-to-surface studies in a massive and automated way.

1. Introduction

With extreme advancement of label-free detection technology, label-free acquisition of images has long been desired in biomedical science because of the capability of providing visual information on native states of cellular and molecular events without label interference [1]. Since the birth of microscopy in late 16th century, numerous label-free microscopy techniques have been developed, such as phase contrast microscopy, differential interference contrast (DIC) microscopy, and more recently surface plasmon resonance microscopy (SPRM). Despite many new features that would have been deemed as impossible at the early time of microscopy, there are still many limitations which prevent label-free imaging techniques from being more widely used. For example, in addition to the perennial difficulty of visualizing function over anatomical structure, it is often quite laborious to spatially localize an event in 3D with good precision.

In this paper, we focus on SPRM to address the 3D localization of an event with an emphasis on extraction of axial information of an image. SPRM relies on the excitation of surface plasmon (SP), which refers to longitudinal electron density waves produced at metal dielectric interface, on a metal (typically, gold) surface with momentum-matched p-polarized light incidence under the well-known SP dispersion relation [2]:

$$k_{sp} = \frac{\omega}{c}\sqrt{\frac{\varepsilon_m \varepsilon_d}{\varepsilon_m + \varepsilon_d}} = k_0 \sin\theta_{in}. \quad (1)$$

Here, k_{sp} and k_0 represent the SP momentum and that of incident photon. θ_{in} is the angle of light incidence. ε_m and ε_d are the permittivity of metal and dielectric ambience. ω and c denote the angular frequency and speed of light in the free space. The momentum-matching condition changes depending on the surface states, including the depth change within the penetration depth of an evanescent wave: as a result, SP resonance (SPR) has been used very successfully as a basis of label-free biomolecular sensors in various formats [3–5]. In an image, the resonance shift as a result of a change in surface states is translated as the variation in reflectivity, i.e., off-resonance appears as an increase of reflectivity. While such a reflectivity changes due to the resonance shift was heavily used for high-throughput analyte detection as imaging SPR [6–8], SPRM itself has been applied to investigating cell–surface interactions [9,10], cell adhesion [11–13], and intracellular organelles [14], and also used to measure membrane protein kinetics and signal transduction [15,16], and to quantify refractive index changes [17] as well as cell–electrode gap [18]. On the other hand, a large portion of the research on SPRM has been devoted to the improvement of the image resolution [19–22]. This is mainly because the propagation length of SP, which is given by

$$L_{sp} = 1/2k''_{sp} \quad (2)$$

with k''_{sp} as the imaginary part of SP momentum, tends to be in the range of 10–100 μm much longer than light wavelength and thus limits practical use of SPRM [23].

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In this work, we have focused more on axial properties of SPRM so that an image may be located more precisely along the depth axis. For this goal, we have taken a comparative approach by which we juxtapose an SPRM image with model resonance characteristics to determine the axial position and separation distance of cell membrane in *in vitro* cell cultures. While such a study was performed by fluorescence interferometry [24], interference reflection microscopy [25,26], and total internal reflection fluorescence (TIRF) microscopy [27], SPRM can provide much improved depth resolution without label interference. In addition, the results may lead to the possibility of extracting axial information massively for better understanding of cell adhesion dynamics and cell-to-surface interactions. The precision of this approach may depend on the validity of a resonance model and has been assessed by considering potential deviations in the model.

2. Methods and materials

2.1. Depth extraction model

The model for depth extraction is illustrated in Fig. 1(a). The model assumes a 7.5-nm thick layer ($d_{mem} = 7.5$ nm) of cell membrane ($n_{mem} = 1.50 \pm 0.04$) between cytosol ($n = 1.36$) and buffer ambiance ($n = 1.33$) [28,29]. The membrane was apart from glass substrate of BK7 ($n = 1.515$) by a distance d, which has been often called separation distance in cell-to-substrate contacts. In effect, the model assumes that the axial distribution of cellular organelles such as cell membrane affects the resonance characteristics dominantly. This can be a valid assumption because SPRM images a structure only within the penetration depth of an evanescent field. Incident light of free space wavelength $\lambda = 632.8$ nm is assumed to remain p-polarized for SPRM. Reflectivity was calculated using Fresnel coefficients.

2.2. Sample preparation

Experimental samples to evaluate the depth extraction model for SPRM were prepared by depositing a 2-nm chromium adhesion layer and a 50-nm gold layer successively on a BK7 glass substrate (No. 1, 18×18 mm^2, Duran Group, Wertheim/Main, Germany) using electron beam and thermal evaporator, respectively. The substrate coverslips were first cleaned with acetone and isopropyl alcohol using a sonicator for 5 min each and then rinsed with distilled water.

2.3. Cell culture

For experimental confirmation, primary human aortic endothelial cells (HAECs) were cultured on the sample substrates. HAECs were purchased from ATCC (Manassas, VA, USA) and cultured in vascular cell basal medium supplemented with endothelial cell growth kit at 37 °C in a humidified 5% CO_2 incubator. HAECs were cultured on the gold film at 1×10^4 cells per dish and stained with 1 μM of CellTrace™ CFSE (Life Technologies, Eugene, OR, USA.) for 20 min at 37 °C for fluorescence imaging.

2.4. Optical setup

An inverted microscope equipped with high NA TIRF objective lens (NA = 1.49, oil immersion, UAPON 100XOTRIF, Olympus, Tokyo, Japan) was used for the SPRM set-up shown in Fig. 1(b). A 20 mW He–Ne laser ($\lambda = 632.8$ nm, 05-LHP-991, Melles Griot, Carlsbad, CA, USA) was used as light source, which is attenuated by a ND filter (FW2AND, Thorlabs, Newton, NJ, USA), p-polarized, and expanded by 20× by a beam expander (GBE20-A, Thorlabs). For comparison, TIRF microscopy (TIRFM) images were also acquired using a 488-nm wavelength diode laser (Obis 488 LS, Santa Clara, CA, USA). The two light sources were combined into a common path by a flip mirror. The light was focused in the back-focal plane by an achromatic lens that is precisely controlled by a linear motor stage (M-UTM150PP.1, Newport, Irvine, CA, USA) up and down along the direction perpendicular to the optical axis. In addition, a pellicle beam splitter (CM1-BP145B1, Thorlabs) was used to reduce ghosting noise. The light waves reflected and scattered by the object on the substrate pass through the beam splitter again and tube lens before they are finally collected by an sCMOS camera (Zyla 4.2, Andor Technology, Belfast, UK).

In order to find SPR dips, the linear motor stage was controlled to adjust light paths so that an angle of incidence through the objective lens can be scanned. The angle of incidence at which the average intensity over a square area (26 μm × 26 μm) of a target cell reaches a minimum was set to be an SPR angle.

3. Results and discussion

Fig. 2 shows the images of target cells acquired by TIRFM and SPRM at different angles of incidence. Compared to TIRFM with fluorescent labels, SPRM suffers clearly from much worse resolution due to SP propagation. Note that even within a single cell, each spatial point may undergo different resonance characteristics thereby different reflectivity. For example, the red and the blue square in Fig. 2(b–e) represent an area of 26 μm × 26 μm, marking a different region in a cell. The squares show that the reflectivity changes to a different degree, i.e., cell center in a red square undergoes a minimum reflectivity around $\theta_{spr} = 74^o$ while cell boundary in a blue circle reaches a minimum in reflectivity around $\theta_{spr} = 71^o$. Resonance characteristics experimentally observed in the cell center and the boundary are presented in Fig. 2(f), which is compared with theoretical results. While the data are overall in good agreement, experimental resonance characteristics are broader likely as a consequence of inter-pixel averaging. In an ideal situation with a completely uniform distribution of target and environment, the averaging does not incur broadening in the SPR characteristics. External factors that include aberration in optics and non-planar beam propagation and internal variations such as intracellular distribution of molecules and membrane may cause the characteristics to broaden. With diffraction-limited optics, the broadening would be dominantly associated with internal variation of molecular and membrane distribution. In this sense, the averaging length may effectively define the lateral spatial resolution. In other words, image resolution can be easily improved in the lateral plane by reducing the averaging length. Note also that the non-uniformity in the measured resonance characteristics may be caused by many factors, most notably the distribution of intracellular molecular distribution. If we disregard the non-uniformity, an equally important factor is the axial location of molecules and/or cellular organelles, the distribution of which can be determined by the depth extraction model.

From the depth extraction model presented in Fig. 1(a), resonance characteristics were calculated with respect to the separation distance between cell membrane and surface of a substrate. The relation of the axial separation distance of cell membrane to the resonance angle θ_{spr} was obtained after the data from the depth extraction model were fitted to a rational function based on the Nelder model [30], as presented in Fig. 3. The relation is applied to the topological SPRM image shown in Fig. 4(a) and translated as the axial position of cell membrane as demonstrated in a color map of Fig. 4(b) and in a 3D map of Fig. 4(c). It is shown that cell membrane is close to the substrate, thus with improved adhesion, with a large resonance angle. Note that the relation of the resonance angle vs. the axial separation distance of cell membrane is not linear. For more details, the profiles of resonance angles and separation distances are presented in Fig. 4(d). The results presented by Fig. 4 marked in a gray region suggest that cell membrane make a contact to the substrate with a buffer distance $d = 40$–60 nm. While one may presume that the distance should be in the range of 10 nm or less for a good contact, the distance in fact is known to vary depending on the cell states and the types of cell contacts such as focal and close contacts with