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In Situ Sensitive Fluorescence Imaging of Neurons Cultured on a Plasmonic Dish Using Fluorescence Microscopy

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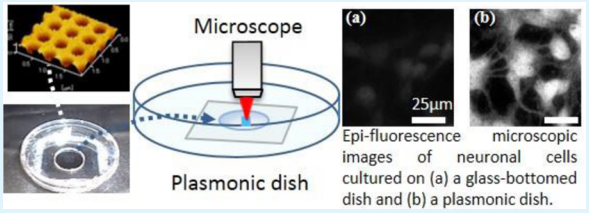
Supporting Information

ABSTRACT: A plasmonic dish was fabricated as a novel cell-culture dish for in situ sensitive imaging applications, in which the cover glass of a glass-bottomed dish was replaced by a grating substrate coated with a film of silver. Neuronal cells were successfully cultured over a period of more than 2 weeks in the plasmonic dish. The fluorescence images of their cells including dendrites were simply observed in situ using a conventional fluorescence microscope. The fluorescence from neuronal cells growing along the dish surface was enhanced using the surface plasmon resonance field. Under an epi-fluorescence microscope and employing a donut-type pinhole, the fluorescence intensity of the neuron dendrites was found to be enhanced efficiently by an order of magnitude compared with that using a conventional glass-bottomed dish. In a transmitted-light fluorescence microscope, the surface-selective fluorescence image of a fine dendrite growing along the dish surface was observed; therefore, the spatial resolution was improved compared with the epi-fluorescence image of the identical dendrite.



Epi-fluorescence microscopic images of neuronal cells cultured on (a) a glass-bottomed dish and (b) a plasmonic dish.

KEYWORDS: *plasmon, culture, fluorescence imaging, grating, microscopy, neuronal cell*

INTRODUCTION

Recent progress in nanofabrication technology has significantly accelerated research into the plasmonic properties of metallic materials. Precise regulation of the size of a metal nanostructure provides a number of possibilities to tailor its plasmonic characteristics. So far, a number of applications have been reported in the literature, such as in optical waveguides,[1] biosensors,[2] solar cells,[3] and bioimaging.[4] The present study was carried out on in situ fine fluorescence imaging based on an enhanced electric field caused by plasmon resonance.

A number of reports on enhanced electric fields using nonmetallic[5,6] and metallic nanostructures[7,8] have been published. In particular, metallic nanoparticles based on localized plasmon resonance have attracted attention,[7,8] because these can provide a marked enhancement in the electric field at a contiguous site using nanoparticles fabricated employing a chemical solution processes. On the other hand, instead of localized plasmon resonance, propagated surface plasmon polaritons are a promising way to make use of enhanced electric fields on a metal surface for exciting a fluorescence molecule.[9] The Kretschmann geometry is a conventional method used to couple the incident light on a metallic thin film coated on the surface of a prism. However, a high incident angle and the complicated optical setup that is required restrict the application of this technique to bioimaging.[10]

Besides the Kretschmann configuration, grating-coupled surface plasmon resonance (GC-SPR)[11,12] has been demonstrated to exhibit an efficient plasmonic enhancement of the fluorescence intensity.[4,13,14] In a fluorescence microscopy, an exposure time can be shortened if an enhanced fluorescence could be collected, so that a photobleaching can be ignored and the kinetics can be also followed. A strong electric field arising from the SPR can be realized under a simple optical setup or a commercially available optical microscope. The advantage of GC-SPR structures is the easy adjustment of the incident angle of the excitation light, which depends on the grating period (Λ). The resonance condition has been described elsewhere[11,12] using the resonance angle (θ_r) and the wave vectors of the surface plasmon polaritons (k_{spp}), incident light (k_{ph}), and grating (k_g) as

$$k_{spp} = k_{ph} \sin \theta_r + mk_g \quad (m = \pm 1, 2, 3...) \tag{1}$$

The value of k_g is proportional to the inverse of the pitch.

In fluorescence microscopic studies, the grating period needs to be considered to utilize the enhanced electric field efficiently based on the SPR, as the resonance angles are included within the illumination angles decided by the numerical aperture (NA) of the objective lens. In an earlier report, we showed that transfected COS cells modified with cy5-labeled green fluorescent protein antibody were dispersed on a one-dimensional (1D) plasmonic chip with a 200 nm-thick silver

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film and were subsequently observed using a fluorescence microscope.[4] The cells were simply observed with a conventional epi-fluorescence microscope and a plasmonic chip, without the complex optical stuff such as Kretschmann-type including a prism[15] and the confocal microscope.[16] The fluorescence enhancement factor, corresponding to the ratio of the fluorescence intensity measured on the plasmonic chip to that on a glass slide, was reported to be more than 20 times higher. Following this, we cultured neuronal cells on a plasmonic chip having a two-dimensional (2D) periodic structure for 2 days, and neuronal cells were observed in situ using a epi-fluorescence microscope.[17] It has been reported that a plasmonic chip with a 45 nm-thick silver film provided an enhancement of more than four times in an analysis considering the mean fluorescence intensities between neuronal cells. The lower enhancement factor for the epi-fluorescence images of the neuronal cells was considered to be from the thinner silver film of the plasmonic chip used (see the Supporting Information). However, in order to study not only epi-fluorescence images but also fluorescence image under the transmitted light illumination, i.e., transmitted-light fluorescence image (Figure 1 (a) and (b)), the plasmonic chip covered

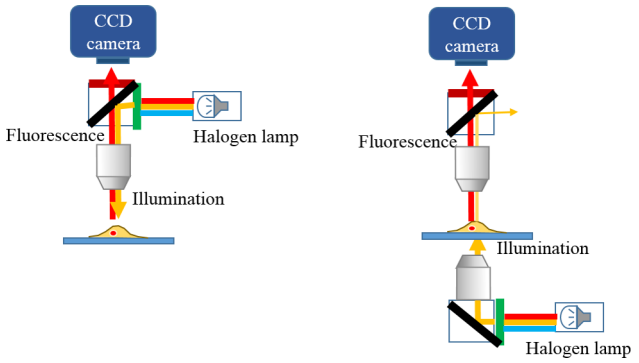

Figure 1. Schematic of (a) epi-fluorescence microscope and (b) transmitted-light fluorescence microscope.

with a thinner 40 nm-thick silver film was required. For the chip coated with 200 nm-thick silver film, the illumination light is little transmitted through a silver film and cannot excite the fluorescence-dye.

In this study, neuronal cells were cultured for a longer period in a 40 nm-thick silver plasmonic dish, which was fabricated by replacing the cover glass of a glass-bottomed dish with a silver grating substrate. Subsequently, the epi-fluorescence and transmitted-light fluorescence images of identical neuronal cells cultured on our novel plasmonic dish were observed in situ under a conventional fluorescence microscope. In order to get brighter fluorescence or larger enhancement factor in epi-mode, the illumination-angle range was controlled. Furthermore, in order to get a fluorescence image of fine dendrites with higher signal-to-noise ratio (S/N ratio), the transmitted-light fluorescence image of neuronal cells was also observed.

EXPERIMENTAL SECTION

Fabrication of the Gratings. A 2D hole−array pattern with a 500 nm pitch and a 30 nm hole depth was fabricated in an area of 4 × 4 mm^2 in the center of a 25 × 25 mm^2 quartz substrate as a mold from NTT-AT. The grating replicas were fabricated usinga UV−nano-imprint method.[18] A UV-curable resin (Toyo Gosei, PAK02-A) was placed on a cover glass (24 × 24 mm^2, Muto Pure Chemicals) modified in advance using silane coupling agent, and then the grating pattern was imprinted using a mold under illumination using a UV lamp (Ushio Spot Cure, 100 mJ/cm^2). The replicas were coated with a thin film using the radio frequency plasma sputtering (rf-sputtering) technique containing (in order) the following: titanium, silver, titanium, and silica layers. The film thickness of the silver, titanium, and silica layers was 36 ± 3 nm, <1 nm, and 30 ± 3 nm, respectively.

Figure 2 shows a representative atomic force microscope (AFM) image of the 2D plasmonic grating with a period of 500 nm. In our

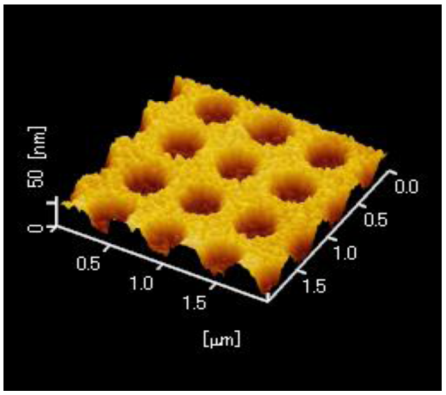

Figure 2. Scanning probe microscopy image of a plasmonic chip.

previous study,[13] a groove depth of 30 ± 5 nm and a duty ratio of 0.5 after metal deposition was found to be preferable for a strong grating coupling with the incident light. The surface roughness (**Ra**), which can strongly influence both the loss in propagation of surface plasmon polaritons and the plasmon enhancement of the electric field intensity, needs to be as low as possible. In this study, we fabricated plasmonic chips with **Ra** ≈ 2 nm.

Cultivation of Neurons on a Plasmonic Dish. A coated grating chip was placed on the bottom of a 35 mm-diameter cell culture dish (glass-based dish, Iwaki) using an adhesive glue[19] (KE-45T, one-component RTV silicone rubber, Shin-Etsu Silicones) instead of a cover glass and left to dry over a period of 24 h (Figure 3). The

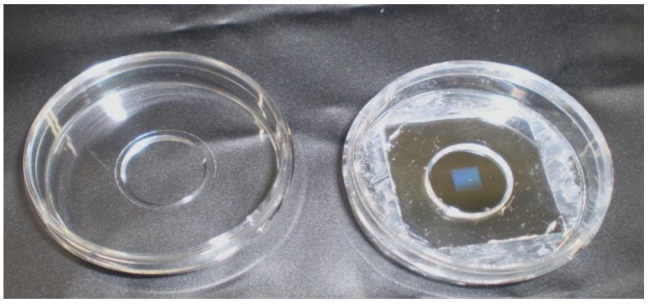

Figure 3. Photograph of the glass-bottomed dish and the plasmonic dish.

following day, the plasmonic dish, the glass-bottomed dish, and the other accessories were sterilized under UV irradiation for 30 min. After coating with polyethylenimine (Sigma-Aldrich),[20] 2.0 × 10^5 of hippocampal neuronal cells separated from the 18-day-old embryos of a Wistar rat were scattered into a 10 mm-diameter cloning ring. They were kept in an incubator maintained at 37 °C in a humidified atmosphere containing 5% CO_2. The cloning ring was removed next day. After culture for 2 days−2 weeks and subsequent formalin fixation, the dish was incubated with Block Ace (DS Pharma Biomedical) for 30 min at room temperature. Immunostaining was performed to fluorescently label the cultured neuronal cells. The primary antibody used was the anti-microtubule-associated protein 2 (MAP2, Sigma-Aldrich) rabbit IgG. The second antibody used was the Alexa Fluor 633-labeled anti-rabbit IgG antibody (Invitrogen).[20]

Finally, the assembly was sealed in a soft mount (Wako) and covered with a 12 mm-diameter cover glass for microscopic observation.

In Situ Fluorescence Imaging. Nerve cells were observed in situ without transforming the cells to the other dish using an upright-inverted fluorescence microscope (BX71, specially made, Olympus), which was equipped with a halogen lamp, an electron multiplying charge coupled device (EM-CCD) camera (Luca R, Andor) and a 40× objective lens (NA = 0.75) for the upright side, and a mercury lamp and a 40× objective lens (NA = 0.6) for the inverted side. Epi-fluorescence microscopic images and transmitted-light fluorescence images were observed under illumination from a halogen lamp and from a mercury lamp, respectively. The EM gain and exposure time were set to 100 and 0.05 s. A donut-type pinhole with a diameter of 0.5−1.5 mm was inserted at the aperture stop (AS) for the upright side to control the illumination angles for the epi-fluorescence imaging. The pinhole restricted the illumination angle of 0−49° to 9−27° (see the Supporting Information Figure S1) in order to improve the contribution of the fluorescence excited by the plasmon-enhanced field compared with that excited by direct illumination. In the transmitted-light fluorescence microscopic images, the illumination-angle range of 0−37° with a 40× objective (NA = 0.6) could cover at least two resonance angles for an individual azimuthal angle (see the Supporting Information Figure S2), because there are two grating vectors with a symmetric axis at Φ = 45° for an individual azimuthal angle on the 2D grating; therefore, an effective coupling between the incident light and plasmon polaritons on the plasmonic dish was expected. Furthermore, the plasmon-coupled emission observed in the normal direction (i.e., 0°) was also effectively collected using these objectives (see the Supporting Information).[21,22]

RESULTS AND DISCUSSION

In Situ Epi-Fluorescence Imaging of Neurons under an Upright Microscope. In our previous papers, the validity of plasmonic gratings was demonstrated for fluorescence imaging.[4,14] In particular, a 2D grating was preferable for brighter fluorescence imaging to a 1D grating because of the efficient 2D coupling of parts of the focused incident light spot on the grating. *S*-polarized light can also couple to a 2D grating. Figure 4 shows an in situ fluorescence image from Alexa Fluor 633-labeled antibody stained to the neuronal cells cultured on a 2D plasmonic dish and on a glass-bottomed dish.

In the epi-fluorescence microscopy images, the excitation light from a halogen lamp operating in the wavelength range 590−650 nm with an appropriate cy5 filter unit was used to illuminate the cells from the upright side. The epi-fluorescence images of neuronal cells cultured for a period of 2 days on the plasmonic and glass-bottomed dishes were observed in situ under the fluorescence microscope after immunostaining. A bright fluorescence image was obtained on the 2D grating, which was 4.6 times brighter than that on the glass-bottomed dish using an open AS (Figures 4(a) and (b)). An enhancement of 4.6 times was nearly the same value as that already reported in our previous paper[17] using a plasmonic chip with almost the same structure. To improve the enhancement factor, a donut-type pinhole was inserted at the AS position and the enhancement in fluorescence improved up to 9.9 times (Figures 4(c) and (d)), although a pinhole decreased an illumination intensity and the total fluorescence intensity was smaller than that in the open AS. The donut-type pinhole restricted the range of illumination angles from the 40× objective to 9−27°. This range covered most of the resonance states for all the azimuthal angles, Φ, and therefore the enhancement in fluorescence was considered to be effectively provided on the plasmonic dish under this condition (see the Supporting Information). About angular dependence of the fluorescence emission, the surface plasmon-coupled emission

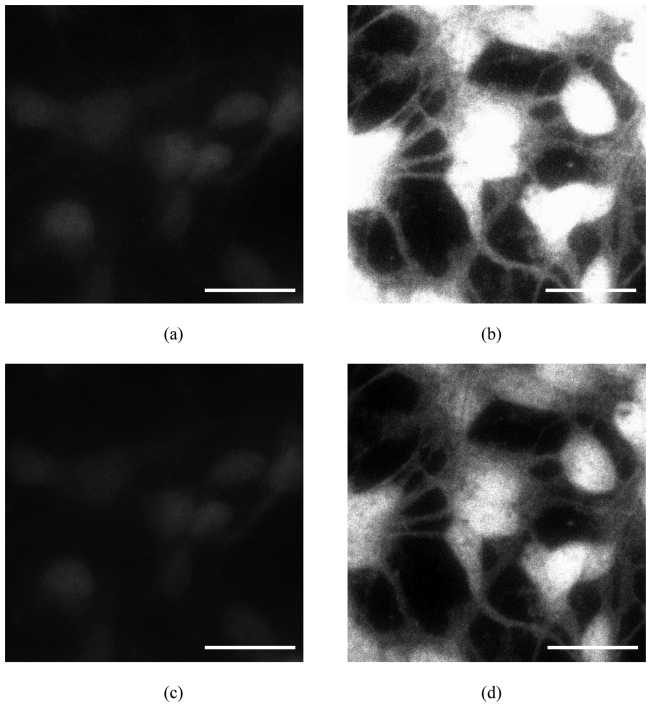

Figure 4. Fluorescence images of neuronal cells cultured on a glass-bottomed dish ((a) and (c)), and on a plasmonic dish ((b) and (d)). (a) and (b) show the images for full open AS; (c) and (d) show images after inserting a donut-type pinhole. All the images are shown using the same contrast, i.e., they were set to 5500 counts for the maximum−minimum values. The bars correspond to 25 μm.

(SPCE) was observed at detection angles close to 0° for all the Φ. Further, the magnification of objective lens used here was not so higher, 40×. Therefore, the images obtained here were not distorted due to the angular dependence of fluorescence.

The contribution of the fluorescence excited by the enhanced SPR field was larger in comparison with that excited by direct illumination. The enhanced electric field based on the SPR induced the enhanced fluorescence from the neuronal cells, as reported previously using a protein pattern.[14] However, a 2D grating for the protein pattern provided an enhancement of 100 times in fluorescence compared with the fluorescence intensity on the glass slide.[14]

The difference in the enhancement factor between these assemblies is considered to be from the following two factors. (i) The approximately 40 nm-thick silver film prepared in this study was thinner than that used in the previous study of 200 nm.[14] The plasmonic dish with a thinner silver film was prepared for use in both the epi-fluorescence and transmitted-light fluorescence microscopy images. In the epi-fluorescence microscopy images, the thick silver film decreased the transmitted light and improved the coupling efficiency compared with the assembly with the thinner silver film. Furthermore, a thicker silver film has larger **Ra** value than the thinner silver film prepared in our laboratory. A larger value of **Ra** increases the fluorescence intensity because of the scattered fluorescence, even on a flat metal surface without a grating. As shown in the Supporting Information, a plasmonic chip with a 200 nm-thick silver film increases the enhancement factor (the ratio of fluorescence intensity measured on the plasmonic grating to that on the glass slide) compared with a 50 nm-thick silver film. An enhancement factor above 20× previously obtained in the fluorescence image of COS cells[4] was also

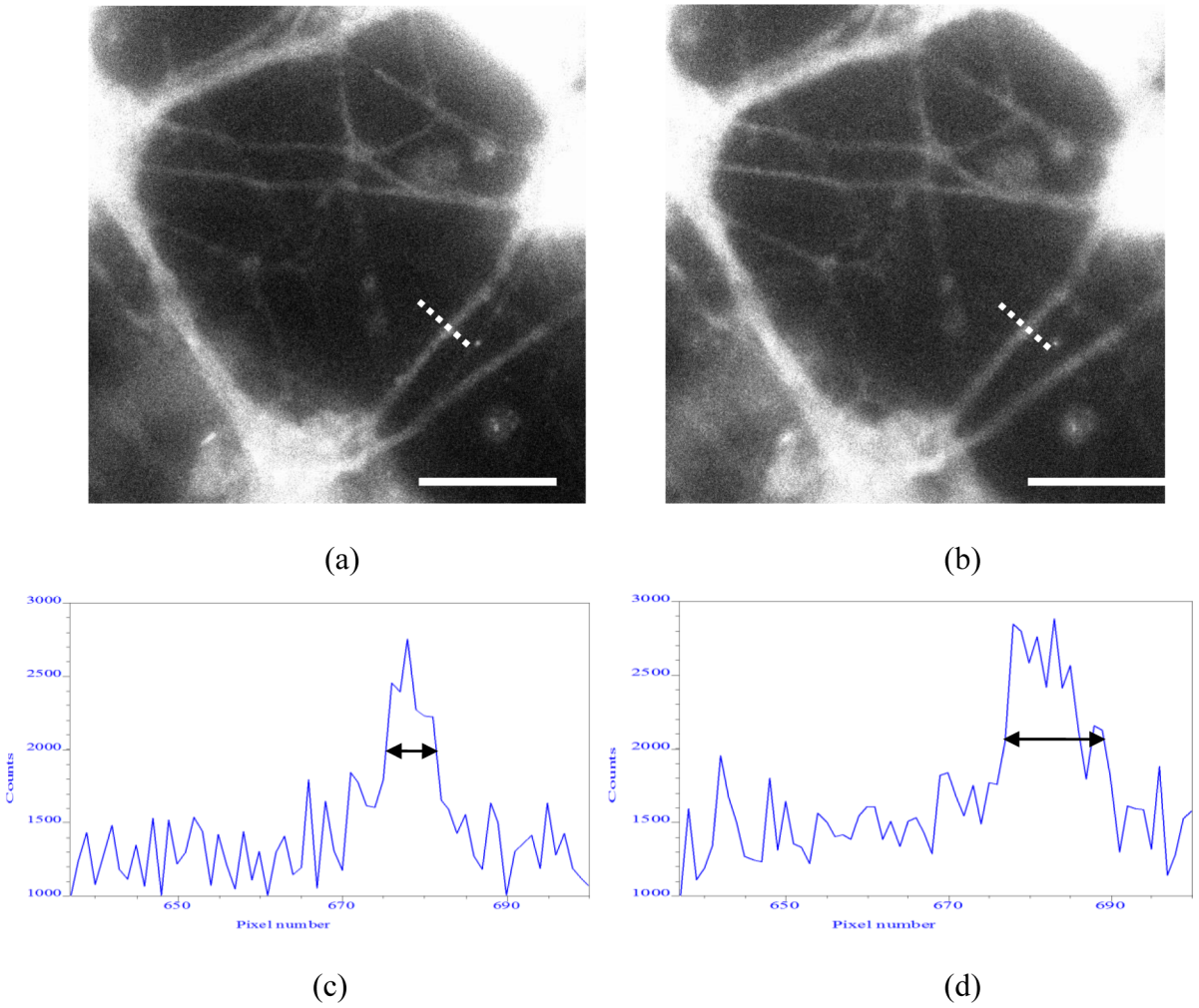

Figure 5. Fluorescence images of neuron cells cultured for 2 weeks on the plasmonic dish observed with (a) a transmitted-light fluorescence microscope and (b) an epi-fluorescence microscope without a pinhole. The bar corresponds to a distance of 25 μm. The width of a dendrite was evaluated from the full width at half-maximum (fwhm) in (c) and (d) and in a cross-section by the broken line drawn in (a) and (b), respectively. One pixel corresponds to 160 nm.

interpreted to be caused by a similar reason. (ii) The other factor is that the thickness of the neuron dendrites is thicker (approximately μm) than the protein layer (approximately 10 nm). Therefore, in the epi-fluorescence images of the cultured neuronal cells, the contribution of the fluorescence excited by the direct illumination, except for plasmons, is not trivial compared with the contribution of the direct illumination in the epi-fluorescence images of a protein array.

In this study, numerous fine neuron dendrites with a width <2 μm were clearly observed (Figures 4(b) and (d)), whereas no such fluorescence images were observed on the glass-bottomed dish (Figures 4(a) and (c)). Such a surface-selective excitation by the surface plasmon field induced a high S/N ratio in the fluorescence images, differing from a general epi-image overlaid along the z-axis. In the fluorescence imaging of for the neuron dendrites, the plasmonic dish could provide in situ brighter image with a higher S/N ratio without removing cells from the culture dish.

In Situ Transmitted Light Fluorescence Imaging of Neurons. Figures 5(a) and (b) show the transmitted-light and epi-fluorescence images of identical neuronal cells cultured for 2 weeks on the plasmonic dish, respectively. In our plasmonic dish, neuronal cells could be cultured for 2−3 weeks. The nanoscale corrugation did not fundamentally influence culture of neuronal cells under the condition of 2×10^5 cells scattered. Cells grew on the grating area and the flat area in an identical dish. Using an upright-inverted fluorescence microscope, two types of fluorescence images of the identical neuronal cells were taken employing an EM gain of 100 and an exposure time of 0.05 s. The original power of the illumination light from a halogen lamp (upright side) was weaker than that from the mercury lamp (inverted side). The backside illumination power decreased because of the 40 nm-thick metal layer, and the fluorescence intensity was, by chance, almost equivalent between the epi-fluorescence and the transmitted-light fluorescence images, as shown in Figures 5(a) and (b).

From an analysis of the cross-sectional areas in the fluorescence images, the width of the fine neuron dendrites was estimated to be 1.0 μm (6 pixels) and 1.7 μm (10.5 pixels) for the transmitted-light and the epi-fluorescence images, respectively. The fluorescence image under transmitted light illumination provided a smaller width close to the real value compared with the epi-fluorescence image under the same observation conditions. It was regarded as an image with a higher spatial resolution due to not the Rayleigh criterion but the higher S/N ratio based on surface selectivity by the

plasmon-field. Fluorescence images were composed of epi-component and plasmon-enhanced fluorescence component. Epi-component occupied in the epi-fluorescence microscopy was larger than that in the transmitted-light fluorescence microscopy. Therefore, in the transmitted-light fluorescence microscopy, the plasmon field-enhanced fluorescence from the neuronal cells attached to the surface was the main contributor, and the fluorescence excited by direct illumination through the 40 nm-thick silver layer provided a contribution of <10%. The plasmonic dish lived up to our expectations for application to long-term cell cultures and for obtaining high spatial resolution images.

CONCLUSIONS

This is the first study that provides simultaneously both in situ epi-fluorescence imaging and transmitted-light fluorescence imaging of neuronal cells cultured on plasmonic dishes. Neuronal cells were cultured for 2−3 weeks in a 40 nm-thick silver plasmonic dish. Despite the fact that the neuron dendrites growing along the dish surface were not as thin as the protein layer thickness, the epi-fluorescence image of a fine dendrite cultivated on the plasmonic dish was around 10 times brighter than that on the glass-bottomed dish. It was due to the effective excitation from the surface-enhanced electric field with a pinhole. Furthermore, in the transmitted-light fluorescence images, a spatial resolution evaluated for a neuron dendrite was higher than that for the identical dendrite by the epi-fluorescence microscopy. The plasmonic dish provided bright epi-fluorescence images and transmitted-light fluorescence images with a high spatial resolution; therefore, this may be a powerful tool for fluorescence microscopic cell observations. In the near future, in situ fluorescence observation of cells under cultivation may also be practical using a plasmonic dish. This will be applicable to various research areas, including cell differentiation or detection of a single protein within a cell.

ASSOCIATED CONTENT

Supporting Information

Details on the reflectivity measured versus the incident angle, the fluorescence intensity measured versus the detection angle, and the relationship between the surface roughness and the fluorescence intensity in the plasmonic chips. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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