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Metal–Dielectric Waveguides for High Efficiency Fluorescence Imaging

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Abstract

We demonstrate that Metal–Dielectric Waveguide structures (MDWs) with high efficiency of fluorescence coupling can be suitable as substrates for fluorescence imaging. This hybrid MDWs consists of a continuous metal film and a dielectric top layer. The optical modes sustaining inside this structure can be excited with a high numerical aperture (N.A) objective, and then focused into a virtual optical probe with high intensity, leading to efficient excitation of fluorophores deposited on top of the MDWs. The emitted fluorophores couple with the optical modes thus enabling the directional emission, which is verified by the back focal plane (BFP) imaging. These unique properties of MDWs have been adopted in a scanning laser confocal optical microscopy, and show the merit of high efficiency fluorescence imaging. MDWs can be easily fabricated by vapor deposition and/or spin coating, the silica surface of the MDWs is suitable for biomolecule tethering, and will offer new opportunities for cell biology and biophysics research.

INTRODUCTION

Surface plasmon–coupled emission (SPCE) has attracted enormous attentions for the past decade.^{1–4} In SPCE, excited fluorophores within the near-field distances from a continuous thin metal film couple with the surface plasmons (SPs) on metal and radiate into a higher–refractive index medium, substrate, with a narrow angular distribution. This kind of directional emission is important in increasing sensitivity; an about 50-fold increase is expected due to high collection efficiency of SPCE as compared to Omni-directional fluorescence. SPCE is possible with two excitation modes; reverse Kretschmann (RK) and Kretschmann (KR). In the Kretschmann (KR) configuration, about 10- to 40-fold-enhanced excitation field should also be considered, which would lead to an overall increase in

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sensitivity of up to 1,000-fold.^{1,2} Additionally, the field depth of the evanescent wave created on the surface using KR excitation, and the distance-dependent coupling of excited fluorophores to the SPs result in high vertical resolution and low background to noise.⁵⁻⁹ These attractive characteristics of SPCE have high implications in sensing applications and expected to bring increased interest on the SPCE based imaging methodologies.¹⁰⁻¹³

For SPCE, due to the high field intensities near metal surface, the closer the fluorophores to the metal surface the higher the enhancement rate for the excitation. However, the fluorophores within a few nanometers from the metallic surface are prone to adverse metal quenching effects. To bypass the disadvantages of metals, a structure consists of multiple dielectric layers called one dimensional photonic crystals (1DPCs) was considered. 1DPCs with appropriate layer dimensions exhibit Bloch surface waves (BSWs), where the optical energy is trapped at the structure – air/sample interface.^{14,15} This phenomenon is conceptually similar to the well-known surface plasmon resonance (SPR) on thin metal films. However the fabrication of 1DPCs is tedious, time consuming and better works only for designed wavelengths due to its photonic bandgap. Another simple approach to alleviate the adverse quenching effects of metals is including a dielectric spacer layer on the metal films used for SP studies. Recently, a metal–dielectric waveguide (MDW) structure that provides high-efficiency coupling of fluorescence at distances over 100 nm away from the metal surface has been reported.¹⁶ Fluorophores on top of the structure efficiently couple into the S-polarized optical mode and radiate most of the energy through the metal film on the glass substrate. The high intensity of the coupled emission is due to combination of effects including an increased excitation field by the prism based KR excitation and efficient coupling of emitted power.¹⁶

In this paper, we demonstrate the suitability of the MDW structure with high efficiency coupling of fluorescence for fluorescence imaging. Fluorescent molecules doped in a PMMA film, that is spin-coated onto the MDW structure with a separation distance over 400 nm from the metal surface, can be excited efficiently by a tightly focused radially polarized beam. The excited fluorophores then couple into several optical modes of the MDW, resulting in a waveguide-coupled emission (WCE). The emitting angle of the WCE is determined by the resonant angles of the optical modes. For comparison, a SP structure containing only SP mode was also fabricated, which will display the SPCE. Polystyrene spheres (PSs) were deposited on both the SP and MDW structures, which work as the objects to be imaged. By using a home built scanning confocal microscopy, we examined the imaging performance of the two structures as the sample substrate and it confirms that MDWs are superior in fluorescence imaging over SP substrates. This result is further corroborated with the back focal plane (BFP) fluorescence images from these two structures. Further we also demonstrated that the MDW shows less energy loss to the metal and hence probes on MDW show more photo-stability. This is because the fluorophores are distant from the quenching metal film. The present MDW is different from previous reports of WCE, which have the fluorophores throughout the dielectric layer.^{17,18} This limits the use of later structures in bio-imaging because the labeled biomolecule are in liquid phase and can't penetrate into the dielectric.

EXPERIMENTAL METHODS

The schematic illustration of the experimental setup and the sample configuration is shown in Fig. 1(a). A radially polarized, collimated laser beam (of 532 nm wavelength) is blocked by an opaque disk in the center to form an annular illumination. The annular beam strikes on the rear aperture of the oil-immersed objective (100 X, N.A, 1.49) and is tightly focused onto the samples. The insert picture in Fig. 1(a) shows the structure of the sample. This MDW substrate was made by magnetron sputtering (Hummer 6.6 RF, Anatech USA). Briefly, 40 nm Ag film was deposited on clean glass slides by sputtering. Then the Ag film was sputtering-coated with 10 (for SP structure) or 390 nm (for MDW structure) thick silica films. The thickness of the layers was determined from the calibration curves of film thickness versus deposition time. The samples were subsequently spin-coated with Rhodamine B (RhB)-doped PMMA film (1% PMMA, 4000 rpm for 1 min), which yields a thickness of 30 nm. The thicknesses of the Ag, silica, and PMMA layers were confirmed with a scanning electron microscopy (SEM). A drop of water solution containing PSs was spread on the multilayer structures (both SP and MDW) and dried for overnight. The diameter of the PSs is about 200 nm. The whole sample was placed on a piezo-scanning stage (PIS) to raster scan the PSs over the focused region of the field. The emitted fluorescence was collected by the same objective, and then focused by a imaging lens (focal length is 400 mm) to the end of a multimode fiber which was connected to a single-photon counter (SPC). The PIS and SPC were integrated to realize scanning confocal imaging. The diameter of the fiber is similar to that of a pinhole, so this configuration can be seemed as a scanning confocal optical microscopy. A sCMOS camera (from Andor) was used to record the BFP image of the objective. The emission spectrum of the doped RhB is presented in Fig. 1(b).

RESULTS AND DISCUSSION

SPCE provides excellent performance in optical imaging due to its attractive characteristics, namely distance-dependent excitation and coupling, high collection efficiency, background suppression and enhanced excitation of nearby fluorophores in KR configuration. Previous report present a new type of MDW structure with fluorophores on the top of the dielectric layer.¹⁶ This MDW structure provides highly enhanced emission compared to SPCE probably due to minimized metal quenching effect. This is because in the MDW substrate the probes located away from the lossy metal surface. We questioned if this kind of MDW structure can perform as a better substrate for fluorescence microscopy than SP substrate do. In order to get a focused optical field as small as possible for the objective based Kretschmann (KR) configuration, we chose radially polarized beam which restricts the excited modes inside the structures to be only transverse magnetic (TM) field.¹⁹

The silica thickness of the MDW structure was set to 390 nm which can sustain two TM modes, TM_1 and TM_2 . The SP structure was also fabricated with 10 nm thick silica film on a 40 nm Ag film. To clearly present the optical modes inside the two designed structures, SP and MDW, we first collected the reflected BFP images from the two structures. The BFP images with SP and MDW substrates are shown in Figs. 2 (a1) and (b1), respectively. To minimize the interference fringes on the BFP images, we used noncoherent 532 nm light

(tungsten bromine lamp combined with a band-pass filter) as the illumination source. The double-headed arrows demonstrate the polarization direction of the illumination beam. The beam fully filled in the rear-aperture of the objective so that the incident angle can range from 0 to 79°. As expected, we can see a pair of dark arcs in BFP image as shown in Fig. 2 (a1), which represents the excitation of SP mode in the SP structure. From the known N.A of the objective, the SPR angle can be derived as 56.13°.

In Fig. 2 (b1), three pairs of arcs were noticed, which correspond to the excitation of TM_1 , TE_2 and TM_2 modes, as labeled in the picture. Figs. 2(a2) and (b2) present the angle-dependent reflectivity from the respective structures, calculated using the transfer matrix method (TMM). In order to take the losses (absorption and scattering due to surface roughness) into account, the following complex refractive indexes were used: $n_{\text{silica}} = 1.46 + i10^{-3}$, $n_{\text{PMMA}} = 1.49 + i10^{-3}$ for silica and PMMA, respectively. The refractive index of the silver at 532 nm was $0.129 + 3.193i$. The calculated resonant angles of all these modes are labelled in the (a2) and (b2), which are consistent with the corresponding angles noticed in the BFP images, (a1) and (b1). Only the TE_1 mode that appeared in Fig. 2 (b2) is not obviously present in the Fig. 2 (b1), which is due to inefficient excitation of the mode, the reflectivity dip of this mode is much shallower than other modes.

It should be noted that the excitation of TE modes in the MDW structure is due to the linear polarization of the excitation beam and axis symmetry of the objective. But if radially polarized beam is used as the excitation source, as shown in Fig. 1(a), only TM modes can be excited, and in the BFP image we could see two concentric dark annuluses, representing the excitation of TM_1 and TM_2 modes (results not shown here). The resonant angle of TM_1 mode is larger than that of TM_2 mode, if we use an opaque circular disk as shown in Figure 1(a), we can block the beam at small incident angle-range and hence forbid the excitation of TM_2 mode. By this kind of excitation, we can realize the excitation of TM_1 mode only. As a result, tightly focused field on the upper surface of the MDW structure can be obtained, which will be used as the optical virtual probe for following BFP fluorescence imaging and scanning confocal fluorescence imaging.¹²

Subsequently, to clearly show the difference in the fluorescence from the two structures, we changed the noncoherent illumination source to a laser beam (with radially polarization) with the same wavelength to excite the RhB molecules. A beam-blocker was also used to spatially filter the laser beam and only to excite the TM_1 mode (of the MDW structure) or SP mode (of the SP structure). The intensity of the excitation laser beam was kept same to easy comparison of the fluorescence efficiency from the two structures. The sCMOS was used to record the BFP fluorescence images. The obtained images from the two structures are shown in Fig. 3(a1) and (b1), respectively. The central wavelength of the fluorescence from RhB is 580 nm, Fig. 1(b), so we used a band pass filter with center wavelength of 580 nm. In Fig. 3 (a1), only one bright ring appears as expected, which indicates the SPCE. While in Fig. 3 (b1), three bright concentric rings are noticed, which correspond to the coupled emission to TE_1 , TM_1 and TE_2 modes, as labeled in the image. Figs. 3(a2) and (b2) shows the intensity distribution along the white dashed-lines in Figs. 3 (a1) and (b1), respectively. For the MDW structure, as shown in Fig. 3 (b2), large fraction of the fluorescence is coupled to the TM_1 mode while a insignificant fraction of fluorescence is

coupled to the TE_1 and TE_2 modes. It is clear from these cross sectional profiles that the WCE of TM_1 , at the resonant angle, is nearly 5-fold higher than that of SPCE.

Next, the sCMOS is replaced with SPC which is integrated with a piezo-scanning stage to measure the following scanning confocal fluorescence images. The polystyrene beads with diameter of about 200 nm objects are deposited on the RhB doped PMMA film and used as the objects for imaging. The confocal images of these beads, deposited on SP and MDW structures are presented in Figs. 4 (a1) and (b1), respectively. The scanning size is $5 \times 5 \mu\text{m}$. In our experiment, these PSs are non-fluorescent and the collected fluorescence signal is from the RhB molecules doped in the PMMA film. From the two scanning confocal images it is clear that the fluorescence signal from areas with PSs on the film is stronger than those without beads, so these PSs can be clearly resolved. The increase in fluorescence intensity might be due to following two reasons. Firstly, for the areas with the PSs on the RhB doped PMMA film, the fluorescence from the RhB molecules emitting into the upper free space may be reflected, or scatted back, into the substrate and thus causing the increase in the collected fluorescence intensity. Secondly, the refractive index of PSs is larger than that of air, so, the excitation of tightly focused, radially polarized laser beam can create strong electric-field near the RhB molecules, between the bead and the PMMA layer. This has been verified by the numerical simulations (data not shown here). The enhancement of excitation field can lead to the incense in the fluorescence signal. As a result, the areas with the PSs show more intense fluorescence than from the areas without the PSs.

To clearly show the details of the scanning confocal images, the white dash-lined boxes in Figs. 4 (a1) and (b1) are enlarged as shown in Figs. 4 (a2) and (b2), respectively. Fig. 4 (a3) and (b3) present the intensity along the white dashed lines shown in Fig. 4 (a2) and (b2). These cross section profiles clearly demonstrate that the fluorescence intensity from the location of the PS on the MDW structure is nearly three times that of on the SP structure. The enhancement factor is not precisely in agreement with the enhancement observed with the BFP images discussed above. This is because in BFP image we are talking about the enhancement at specific angle while in confocal image we are talking about the total fluorescence collected by the objective.

The full width at half maximum (FWHM) of emission spots due to single PS on SP structure, as presented in Fig 4 (a3), is 190 nm, and that on MDW structure is 200 nm, Fig. 4(b3). The resolution of PSs on the two structures is nearly the same, which demonstrate that MDW structure can accomplish high efficiency fluorescence imaging without loss of resolution. It is noticed that the shape of the PSs images presented in Figs. 4(a2) and (b2) is not strictly circular in symmetry and there exists a 10 nm distortion. We believe this discrepancy might be caused by other small scatters which tend to gather around the PS. It should be noted that, in our experiment, the PS objects used for imaging were not labeled with any fluorophores, rather fluorophores were doped in the substrate (PMMA layer). Accordingly, we anticipate that this approach can be useful as a label-free imaging method.²⁰

It should be noted here that, unlike freely diffusing probe in solution, the probe immobilized in rigid matrix such as in PMMA shows significant photo bleaching. Further, photo

bleaching or probe stability in PMMA can be environment sensitive. For this reason it is reasonable to compare the fluorescence intensity changes from SP and MDW structures vs the illumination time. Additionally, it is a known fact that metals at short fluorophore–metal distances, due to energy dissipation to the lossy metals, can cause the fluorescence quenching and rapid photo-induced redox reactions.²¹⁻²³ By placing the fluorophores at distances of about 400 nm away from the metal surface, these effects of metal can be reduced. To compare the difference in these effects on the fluorophores on the two structures, we measured the fluorescence intensity versus illumination time. In this case, the laser beam was focused onto a fixed spot on each sample and then recorded the fluorescence intensity. To have a meaningful results, we have recorded the intensity vs. illumination time profiles at 5 different positions on each structure and averaged data has been used to get the curves shown in Fig. 5. The fluorescence intensity from the two structures consistently reduced with the increased illumination time. But impressively, fluorophores on MDW structure shows higher stability than that on the SP structure. It takes about 10 seconds for the fluorescence to decay away to half its original level of intensity on SP structure, while that on MDW structure it takes about 100 seconds. This indicates that the effective emission time of fluorophores on MDW structure is about 10-fold longer than that on the SP structure.

CONCLUSION

In summary, a MDW structure with high efficiency of coupled fluorescence emission was fabricated and used as the sample substrate for fluorescence microscopy. This MDW structure sustains several guided modes mainly distributed in the dielectric layer. By a focused radially polarized annular beam illumination, a virtual optical probe is formed in the dielectric layer. Fluorophores on top of the structure were excited by the virtual optical probe and then coupled into the optical modes inducing the directional and enhanced fluorescence emission, which is verified by fluorescence BFP image. By scanning the sample with the virtual optical probe, confocal fluorescence images assisted by PSs on the substrate is achieved. In contrast to the structure used for SPCE, MDW substrate provides highly enhanced fluorescence image. Also, due to minimized metal quenching effects in the MDW substrate, the fluorophores from the MDW structure are more stable, over 10 times longer, than that from the SP structure. This simple MDW design can be readily modified for different resonant wavelengths by changing the thickness of the dielectric layer. The multilayer format offers new opportunities for high efficiency fluorescence imaging.

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Fig 1.
 (a) Schematic configuration of the experimental setup. The insert shows the structure of sample. (b) The coupled emission spectrum from RhB molecules.

Fig 2.

Reflected BFP images of SP (a1) and MDW (b1) structures. The wavelength of the illumination light is 532 nm, the double-headed arrow line represent the polarization of the incident light. Calculated angle-dependent reflectivity from the SP (a2) and MDW (b2) structures.

Fig 3.

Panels (a1) and (b1) are fluorescence BFP images from SP (10 nm SiO₂) and MDW (390 nm SiO₂) structures at 580 nm wavelength, respectively. Panels (a2) and (b2) are the cross-section profiles of the intensity along the white dashed lines shown in (a1) and (b1), respectively.

Fig 4. Scanning confocal fluorescence images of individual PS on the two structures, SP (a1) and MDM (b1). Panels (a2) and (b2) are enlarged confocal images of the white dash-lined boxes labeled in (a1) and (b1). Figures (a3) and (b3) are cross-section profiles of the intensity along the white dashed lines in (a2) and (b2).

Fig 5.
Fluorescence intensity from SP and MDW structures with illumination time.