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Two-photon fluorescence excitation using an integrated optical microcavity: a promising tool for biosensing of natural chromophores

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Abstract

Application of an integrated optics (IO) microcavity (MC) for evanescent excitation of two-photon excited fluorescence (TPF) is demonstrated. The MC provides a high local intensity, which is required for the TPF, because of resonant enhancement of the intracavity power and a strong two-dimensional confinement of the guided mode. Numerical estimations show a large increase, by more than a factor of 10^4 of the TPF intensity at the MC compared to a conventional straight waveguide. This will lead to a significant improvement of the detection limits of UV-absorbing chromophores (down to 10^{-8} M) when using the MC as a biosensor. Feasibility of TPF excitation using an IO MC is confirmed experimentally for the first time.

Keywords: Integrated optics; Microcavity; Biosensor; Two photons; Fluorescence

Integrated optics (IO) microcavities (MCs) have been extensively investigated in recent years [1–4]. Advances in microfabrication led to the realization of IO MCs as small as several micrometers in radius supporting resonances of high Q in excess of 10^4 [3,4]. Due to resonant enhancement of the optical field inside an MC, the efficiency of many (non)linear optical processes such as absorption, scattering and emission is increased [5]. IO MCs have been proposed for the use in optical networking [1,2], low threshold microlasing [6] and electro-optic modulation [7]. Motivated by fundamental and technological advantages of IO MCs, we have recently suggested their application to resonant-enhanced refractive index sensing [8], evanescent-wave spectroscopy [9] and combination of multiple MC sensors into an array [10]. We have shown that the MCs are advantageous for (bio)sensing because of: (i) small sensing volume of the MC, which requires only a few femtoliters of the sample and (ii) resonant-enhanced sensitivity that is directly proportional to the Q of the MC.

In these studies [8–10], conventional one-photon absorption/fluorescence has been employed. Two-photon absorption/fluorescence (TPA/TPF) has not been combined with an IO MC until now. Yet, TPF is an important method in evanescent-wave MC-based (bio)sensing since it allows one to: (i) increase the signal-to-background efficiency of evanescent excitation because TPF quadratically depends on the incident power and (ii) access UV-absorbing fluorophores with visible laser excitation. A prerequisite for observing a detectable TPF signal is high excitation intensity, typically, in excess of $>10^5$ W/cm^2 [11]. An IO MC, as a high-Q resonant structure, enables the enhancement of the incident power at resonance and, thereby, promotes TPF excitation. In this paper, we present an estimate for the efficiency of TPF excited with an IO MC and show the experimental feasibility of the MC-assisted TPF.

TPF is a process in which two excitation photons are annihilated simultaneously by a molecule, and subsequently, the molecule emits a fluorescence photon at a wavelength smaller than that of the excitation. Using the basic theory of TPF [12] the time-averaged flux of TPF generated from a fluorophore

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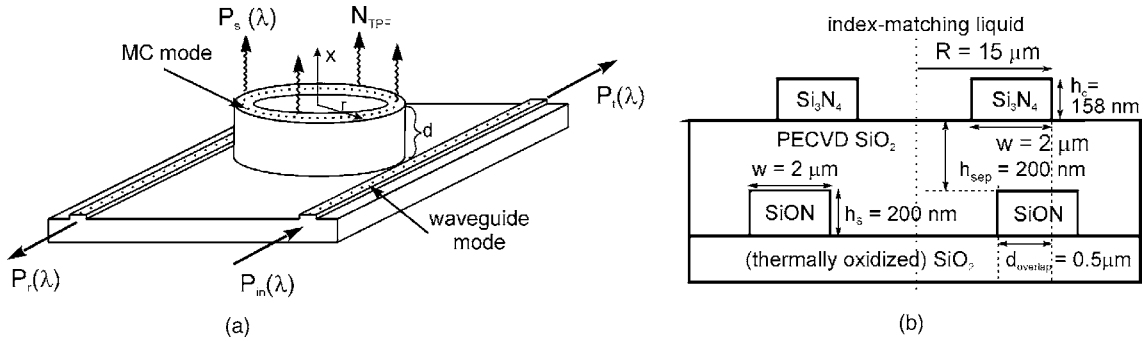

Fig. 1. (a) Perspective view of an IO MC used for TPF excitation. Light from a tunable (pulsed) laser source ($P_{in}(\lambda)$) is launched into the waveguide, which excites a MC mode. The second (optional) waveguide is used to probe the power ($P_r(\lambda)$) inside the MC. The transmitted ($P_t(\lambda)$) and scattering ($P_s(\lambda)$) signal is measured to identify MC resonances. An analyte solution (tryptophan dissolved in glycerol) is put on top of the MC and the TPF signal N_{TPF} is measured from the top. (b) The cross-section of the ring MC coupled to the MC used for TPF experiments. The MC was fabricated in the SiON technology as described in [4]. Refractive indices: $n(Si_3N_4) = 2.00$, $n(SiO_2) = 1.47$, $n(SiON) = 1.55$. As a cover medium, glycerol ($n = 1.47$) was used.

excited by the MC of radius R (Fig. 1a) is calculated as:

$$N_{TPF}^{MC} = 0.5 q_F \frac{g_p}{f\tau} C\sigma^{(2)} L_{MC} \left(\frac{P_{MC}}{A}\right)^2 \int_d^\infty \int_0^\infty dx dr S_{MC}^2(x, r) \quad (1)$$

where: q_F, C and $\sigma^{(2)}$ are the quantum yield, the concentration and the two-photon absorption cross-section of the fluorophore, respectively; τ and f are the pulse length and repetition rate of the laser source exciting the MC, g_p is a numerical constant accounting for the pulse shape (e.g., $g_p = 0.588$ for a hyperbolic-secant square pulse); P_{MC} is the time-averaged intracavity power, A is the effective cross-section of the guided mode of the MC and $S_{MC}(x, r)$ is the 2-D intensity profile of the MC mode. It is explicitly assumed in Eq. (1) that the fluorophores are excited by the whole evanescent volume of the MC, that is, $d < x < \infty$, $0 < r < \infty$ and $L_{MC} = 2\pi R$. There are two factors that make the MC superior to a straight waveguide for TPF excitation. First, at resonance, the intracavity power P_{MC} exceeds the incident power P_{in} in the adjacent waveguide (Fig. 1a) by a factor of G:

$$G = \eta G_m = \eta \frac{\lambda Q_0}{4\pi^2 R N_{eff}} \quad (2)$$

where η is the extraction efficiency of the incident power from the waveguide, which is equal to 1 in case of critical coupling [10]; Q_0 is the intrinsic quality of a resonant mode [4] of the MC and N_{eff} is the effective refractive index of that mode [4,10]. A technologically feasible MC may have a Q_0 of 10^5 (e.g. [4]) with $R = 15\,\mu m$ and $N_{eff} = 1.7$ [4,8]. Then, at $\lambda = 580\,nm$, the enhancement of the TPF at the MC due to the resonance effect is equal to: $G^2 = 0.3 \times 10^4$. Second, a guided mode of a ring or disk IO MC is better confined laterally (along radius r, Fig. 1a) as compared to a straight waveguide mode (ca. 0.6 μm for the MC [4,10] and >1 μm for the waveguide). Better confinement of the mode results in smaller A (Eq. (1)) and, therefore, in higher TPF intensity per fluorophore molecule.

Here the performance of the MC for TPF excitation is estimated. We have done this for tryptophan (Trp), an amino acid that is a natural, fluorescent component in proteins. Trp has

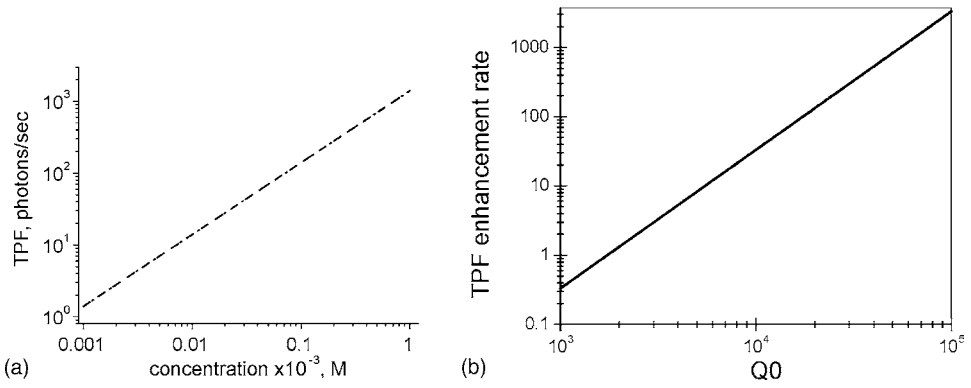

Fig. 2. (a) The estimated TPF intensity from tryptophan excited with the waveguide of a length $L = 2\pi R = 94.2\,\mu m$ as a function of the fluorophore concentration dissolved in glycerol ($n = 1.47$). Experimental conditions: detection efficiency = 0.01, $\lambda = 580\,nm$, $P_{in} = 30\,\mu W$, $\tau_p = 8\,ps$, $f = 4\,MHz$. (b) Relative TPE fluorescence intensity generated from tryptophan in the evanescent volume of the MC as a function of the intrinsic Q_0 of the MC. The fluorescence signal is normalized to that excited by the waveguide of $L = 2\pi R$, where R is the MC radius.

been recognized as one of the key fluorescing species that accounts for up to 90% of the fluorescence of proteins from various tissue and cell samples [13]. Trp possesses a pronounced absorption band in the UV region around 270–290 nm. Therefore, efficient two-photon absorption/fluorescence of Trp should occur when excited in the range of 550–580 nm. These excitation frequencies are supported by the MC and readily available with commercial pulsed dye lasers. First, the TPF rate generated from Trp in the evanescent volume of the MC with $G = 1$ was estimated. Such a case corresponds to the TPF excitation using a straight waveguide of a length $L = 2\pi R$ and assuming equal spatial integrals of Eq. (1) for the waveguide and the MC. The result of calculations of N_{MC}^{TPF} as a function of the fluorophore concentration is shown in Fig. 2a. The data of the graphs suggest that a fairly intense flux of TPF photons (>10^2 photons/s) can be observed even for relatively low concentrations used (<10^{-4} M). An estimation of the detection limit can be made on the basis of the detector performance. For instance, if an avalanche photodiode is used for fluorescence detection, the dark count rate can be as low as 25 counts/s. For a signal-to-noise ratio of unity the detection of $\sim 2 \times 10^{-5}$ M appears feasible. Second, the TPF rate from the MC at $G > 1$ was calculated. The resulting dependence of MC-induced relative TPE fluorescence rate versus the intrinsic Q_0-factor of the MC, calculated for the case of critical coupling ($\eta = 1$, Eq. (1)) is shown in Fig. 2b. Evidently, the MC-excited TPF starts to dominate that from the waveguide as soon as the Q_0 exceeds 2000. At $Q_0 \cong 10^5$, enhancement of TPF rate at the MC allows dramatic reduction of the fluorophore concentration by up to four orders of magnitude compared to straight waveguide excitation. It means that detection of as low as 10^{-8} M of Trp should be feasible by means of the MC.

Experimental study of TPF excited by an IO MC was performed using a ring MC (Fig. 1b). First, the resonant properties of the MC were characterized by measuring its normal scattering ($P_s(\lambda)$) and transmission ($P_t(\lambda)$) spectra (Fig. 1a) excited by a pulsed tunable laser. A series of periodic resonance peaks in the scattering with complementary dips in transmission was detected as the excitation wavelength was scanned (Fig. 3). A free spectral range (FSR) observed (ca. 1.75 nm) correlates reasonably with a calculated FSR of 2.1 nm. This indicates that the laser pulses can couple efficiently to MC resonances, as in the case of CW excitation [9]. The MC resonance near 583.2 nm, which has a Q of 1630 ± 136 and a relative transmission dip of about 10%, was used for resonant excitation of the TPF. The intracavity power P_{MC} associated with this mode was estimated (using the approach described in [10]) to be $P_{MC} = 0.30 P_{in}$, where P_{in} is the power in the adjacent waveguide. Despite of low $G \ll 1$ (Eq. (2)) for the MC used, a considerable amount (10%) of the pulsed energy can be still transferred to the MC at resonance and can be used for TPF excitation.

A solution of an UV-absorbing fluorophore, tryptophan (Trp), was applied on top of the MC to observe the TPF. The Trp solution was covering the MC itself and the area

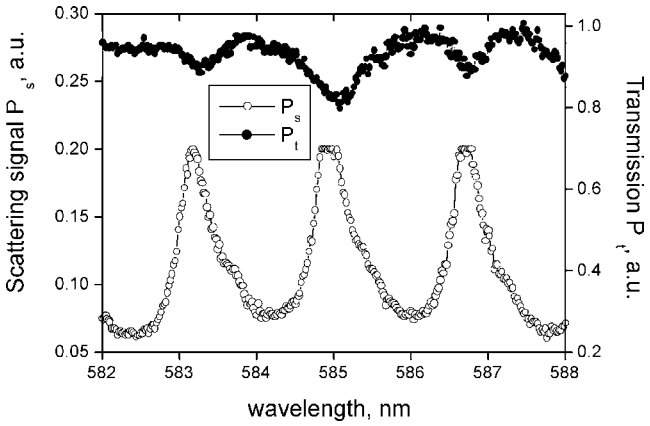

Fig. 3. Scattering ($P_s(\lambda)$) and transmission ($P_t(\lambda)$) spectra of the ring MC (Fig. 1b excited with a pulsed cavity-dumped tunable Rhodamine 6G dye laser (700 Series, Coherent) pumped by a mode-locked Nd-doped YLF laser (Antares 76-YLF, Coherent). The transmission curve is normalized to the transmitted power out-of-resonance. The very top of the resonance peak near 585 nm is saturated due to a detector.

of the waveguide next to it (Fig. 1a). With the Trp applied, the scattering and transmission spectra are virtually the same as those in Fig. 3. By adjusting the laser wavelength, the MC was excited near the resonance maximum at 583.2 nm (Fig. 3). The photons of this wavelength are efficiently absorbed in a two-photon process by Trp, whose absorption band is found within 270–300 nm range [13]. As a result, the TPF of Trp is induced in the range from 300 to 450 nm [13]. A UV image of the MC covered with a Trp solution and excited at the 583.2 nm resonance is shown in Fig. 4a. Clearly, the UV emission is excited both by the waveguide and the MC. When the excitation wavelength is tuned out of the resonance to 584.2 nm (Fig. 3), the UV emission from the MC area disappears (Fig. 4b). At the same time, the emission from the waveguide area remains unchanged, because the power P_{in} in the waveguide does not alter. Therefore, it is the excitation of Trp by the resonant mode of the MC at 583.2 nm that gives rise to the UV emission observed from the MC. In order to prove further that the observed UV emission was due to the TPF of Trp, the UV signal dependence was analyzed. Instead of measuring a quadratic dependence of the TPF on the excitation power [11,12] we analyzed the UV intensity as a function of the reciprocal pulse repetition frequency $1/f$ (Eq. (1)) keeping the time averaged power P_{in} and the pulse length τ constant. Fig. 5 shows the pixel-averaged fluorescence intensities collected from the waveguide (Fig. 5a) and the MC (Fig. 5b) as a function of $1/f$. For both the waveguide- and the MC-generated signal a linear dependence is observed. This implies, according to Eq. (1), that the UV emission observed from the MC (Fig. 5b) is indeed due to the TPF of Trp.

The TPF intensity from the waveguide area was observed to be superior to that from the MC area (Fig. 4). Apparently, the absolute power inside the MC (P_{MC}) is lower than that carried by the adjacent waveguide (P_{in}). This is a result of poor coupling ($\eta \ll 1$, Eq. (2)) between the waveguide and the MC and relatively low Q of the used MC device, which is

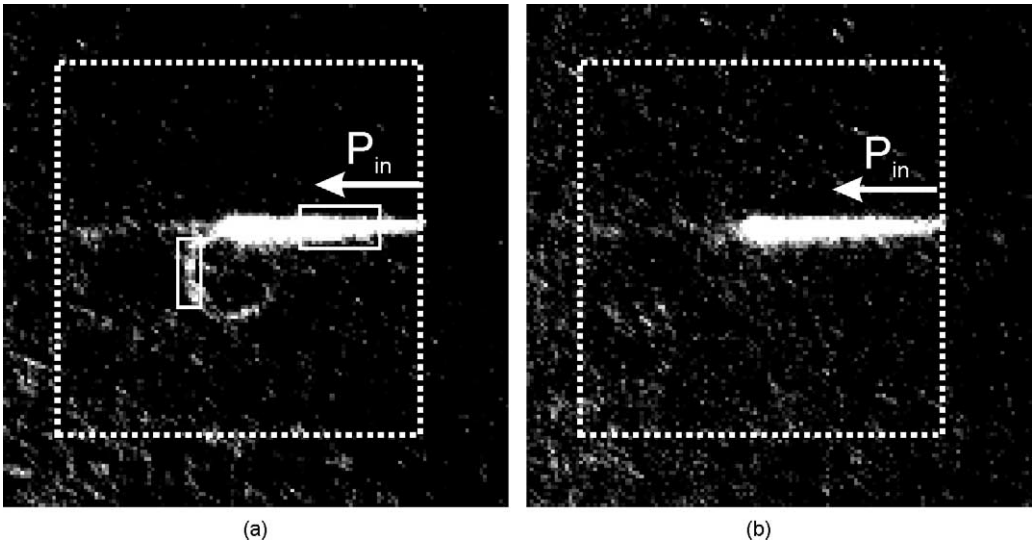

Fig. 4. UV images of the MC and the adjacent waveguide (as in Fig. 1a covered with the glycerol solution of Trp (ca. 15 mg/ml) when the MC is on-resonance at 583.2 nm (a) and off-resonance at 584.2 nm (b). The dotted rectangle shows the boundaries of the etched window around the MC and the waveguide that is filled with the Trp solution. Outside the window no TPF can be excited because of a thick layer of SiO_2 covering the wafer. The scattered light from the MC is rejected using a notch filter. The UV emission of Trp is spectrally selected by a bandpass color glass filter (350–400 nm). The images were recorded using a N_2-cooled CCD. Excitation conditions: $\tau_p = 8$ ps, $f = 4$ MHz, $P_{in} = 0.4$ mW, integration time = 3 s.

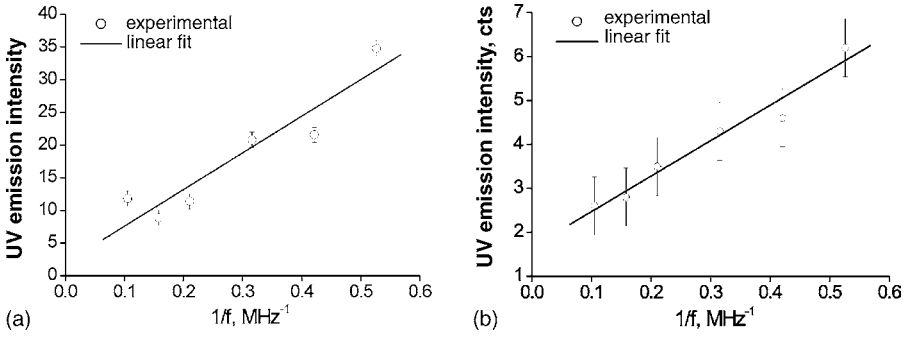

Fig. 5. Average TPF intensity analyzed from the image in Fig. 4a from the waveguide area (a) and MC area (b) as a function of the reciprocal pulse repetition frequency 1/*f*. The areas of the waveguide and the MC images from which the signal is integrated are shown by the rectangles in Fig. 4a.

confirmed by the spectra in Fig. 3. Moreover, the TPF signal observed from the waveguide before the MC/waveguide coupling region is more intense than that from the waveguide area behind the MC (Fig. 4). This is due to high insertion losses at the point of closest proximity of the MC to the waveguide. The results obtained clearly show the feasibility of resonant-enhanced TPF using an integrated optical MC. Optimization of the quality and the coupling of the MC to the waveguide will result in an increased TPF of the MC versus the waveguide. In conclusion, we demonstrated, both theoretically and experimentally, the feasibility of TPF excitation using an IO MC. The results of the study are particularly important for development of IO MCs for resonant-enhanced two-photon (bio)sensing. Detection of 10^{-8} M of UV-absorbing fluorophores (tryptophan) appears feasible with the state-of-the-art technology. In addition, the effect of TPF provides a natural means for the extension of waveguide technologies to studies UV-absorbing fluorophores, especially intrinsic ones such as NAD(P)H, tryptophan, serotonin, etc. High resonant-enhanced sensitivity of the TPF method based on an IO MC is of particular advantage for studying these natural chromophores compared to both waveguide and bulk excitation schemes. Finally, a MC is built using conventional waveguide materials and design tools. Therefore, MC-assisted TPF can be combined with a wide variety of sensing approaches as immunoassay or any other surface-recognition based techniques.

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