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Evanescent-field-induced two-photon fluorescence: excitation of macroscopic areas of planar waveguides

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ABSTRACT Two-photon excitation (TPE) of fluorescence is a powerful tool for separating a faintly emitted fluorescence signal from background excitation noise. Until now TPE has only been accomplished for very small excitation areas of a few square micrometre dimensions since they are readily available in the focal zone of high-power lasers. In this paper we demonstrate, to our knowledge for the first time, two-photon excited fluorescence with planar thin-film waveguide structures of macroscopic excitation areas of the order of square millimetres to square centimetres.

Based upon our results, it can be envisaged that the new combination of planar waveguide technology with TPE will become a powerful tool for macroscopic surface-interaction studies. It can also open the way for further improving the sensitivity of biosensing platforms like genomic and proteomic microarrays based upon planar waveguides.

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1 Introduction

Two-photon excitation (TPE) of fluorescence is a process in which two photons are simultaneously absorbed to arrive at an excited state of a molecule through a virtual state. The up-converted fluorescence usually leads to efficient rejection of background noise and hence allows detection of faintly emitting traces – provided they are accessible by two-photon excitation [1]. TPE requires high photon densities, i.e. intensities in excess of 10^5 W/cm^2 [2]. Therefore, this technique has been used only for excitation areas of the size of the order of the diffraction-limited focus of high-power lasers.

For the study presented here, thin-film planar waveguides are used as the sample geometry. High refractive index planar waveguides have been introduced as sensitive transducers for fluorescence-based (bio)sensors in the last decade [3, 4]. Based upon this technology, detection sensitivities on macroscopic sensor areas could be improved by about two orders of magnitude when compared to fibre-optic sensing configurations or to confocal scanning systems [5]. The origin of this increase in sensitivity is the confinement of the energy of the guided wave to a sub-wavelength-dimension cross section, combined with specific interaction between the analyte molecules and surface-bound selective recognition elements immobilised on the active face of the waveguide. For achieving ultra-low detection limits in the femtomolar range when using visible excitation light, monomode waveguides with thicknesses ranging from 100 nm to 200 nm and a refractive index of 2.0 or higher, deposited on a transparent non-fluorescent substrate like glass, are used. For waveguides with these parameters, 10% and more of the maximum intensity of the guided light is available in the zone of the evanescent field for interaction with the analyte. The intensity of this field decays exponentially with the distance from the waveguide. Due to the perfect confinement of the guided excitation light to the waveguide and only within its immediate proximity, very high excitation intensities are available on and close to the surface of the planar waveguide. These excitation intensities are orders of magnitude larger than in conventional epi-fluorescence configurations (with non-focused beams) and can reach the values achieved in focused laser beams. As a significant advantage compared to confocal fluorescence-detection configurations, these strong excitation fields are not only available in a microscopic focus, but along the whole surface of the waveguide where the excitation light is guided. In addition, the bulk medium on top of the waveguide layer is not excited, i.e. interference from the bulk medium can be discriminated with high efficiency.

Upon replacing the rather weak excitation-light sources typically used for biosensing applications by more powerful lasers, a combination of two-photon excitation with planar waveguide fluorescence technology becomes possible. This opens the way for the development of a new generation of (bio)sensors and of platforms for bio-analytical microarrays with improved sensitivity on one hand, and for the implementation of less-complex instrumentation for the study of two-photon excitation processes on extended surfaces on the other hand. In the following, we present for the first time planar

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two-photon excitation on macroscopic areas of several square millimetres on planar waveguide structures with two grating couplers. This increase of the interaction volume by orders of magnitude is based on the excitation-field enhancement along the planar waveguide and on spatial confinement to its surface.

2   Experimental configuration

The waveguide structure (Unaxis Balzers, Liechtenstein) consists of a 150-nm thin-film layer of Ta_2O_5 (refractive index $n = 2.092$ at 800 nm) on a glass substrate ($n = 1.496$ at 800 nm), equipped with two coupling gratings of period 360 nm, depth 12 nm, and length 0.5 mm, placed at a distance of 8.5 mm apart from each other. A drop of rhodamine B solution (1.59×10^{-5} M) in ethanol was deposited on the surface of the Ta_2O_5 layer in between the two gratings. After evaporation of the solvent, the rhodamine molecules remained immobilised on the waveguiding layer, covering about half of the area between the gratings. The dye rhodamine B is well described in the literature [6, 7] for its TPE properties.

An 80-MHz mode-locked titanium:sapphire laser operating at 800 nm with a spectral bandwidth of about 8 nm served as the excitation source. The incident pulse duration at the coupling grating was 150 fs. The maximum average power at the coupling grating was 600 mW, corresponding to 7.5-nJ pulse energy with 44-kW peak power. The power could be continuously adjusted by a computer-controlled acousto-optic modulator (AOM). The AOM was also used to chop the light beam for lock-in detection of two-photon fluorescence signals. The excitation beam shape on the in-coupling grating could be controlled by using different lenses in the optical light path. Light emanating from the waveguide area between the gratings was collected by a lens system and imaged either on a digital camera for laterally resolved detection, or focused onto a GaAsP photodiode for detection of a single spatially integrated signal. Filters could be inserted into the optical path of the emission light in order to discriminate against the light at the launched excitation wavelength.

3   Results and discussion

In a first experiment, the laser beam was slightly focused to a 100-μm beam waist and aligned at the coupling resonance angle onto the in-coupling grating. Figure 1 (upper part) shows details of the Ta_2O_5 chip equipped with the two grating couplers. The lower part of this figure displays the image of the fluorescence trace (without IR-blocking filter). The scattered light from the coupling gratings is clearly resolved. Inspection by the naked eye shows the yellow fluorescence of the immobilised rhodamine molecules, whereas no emission is observed from the region without rhodamine B coverage. It should be noted that significant attenuation of the observed fluorescence signal along the guided mode cannot be observed. In addition, the area of two-photon fluorescence emission is identical to the area of the guided radiation inside the waveguide, covering a surface of up to 3 mm in width and 7 mm in length; hence exceeding an excitation area of 20 mm^2.

A GaAsP photodiode was used as the detector in order to determine the accurate dependence of the observed emission intensities on the excitation-light intensity. The incident laser power was measured by a Si photodiode placed in the beam reflected from the in-coupling grating. In order to reject the environmental light and to increase the signal-to-noise ratio, the incident light beam was chopped (2 kHz) and the signals were detected with a lock-in amplifier. Figure 2 shows the measured fluorescence intensity as a function of the incident laser power. The excellent fit to the data by a pure quadratic dependence is clear evidence that no linear absorption or higher-order processes occur. We conclude that the observed fluorescence of rhodamine B can unambiguously be attributed to two-photon absorption. Under the applied conditions, the observed emission is free from any additional background signals. The absence of an observable light signal along that part of the propagation path between the in-coupling grating and
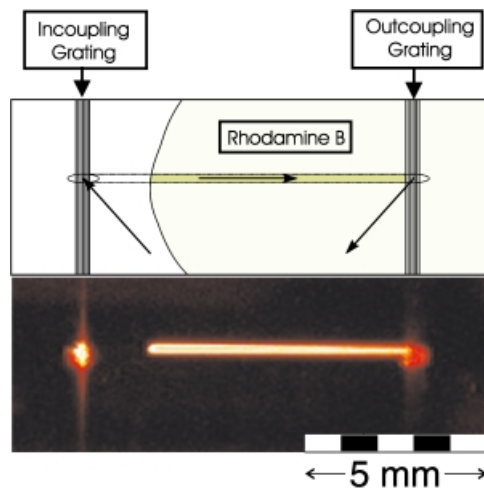
FIGURE 1   Schematic of waveguide structure with coupling gratings, guided beam path, and location of immobilised rhodamine B (*upper part* of figure); image of fluorescence trace (without IR-blocking filter). Scattered light from in- and out-coupling gratings is clearly resolved. Inspection by the naked eye shows yellow colour of the fluorescence of the immobilised rhodamine B molecules, whereas no emission is observed from the region without rhodamine B coverage (*lower part*)
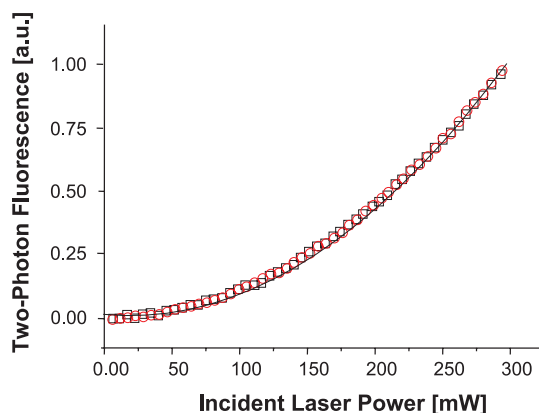
FIGURE 2   Two-photon fluorescence intensity dependence on the incident laser power showing pure quadratic dependence: clear evidence for the prevailing second-order process

the onset of the region occupied by the immobilised rhodamine molecules is in addition a clear indication that other non-linear processes like surface-confined second-harmonic generation can be excluded as reasons for the observed emission.

It should be pointed out that the major effect responsible for the apparent intensity enhancement within the waveguide – leading finally to the observed TPE fluorescence – is the compression of the excitation cross section from 0.5 mm to 150 nm in one dimension due to the coupling grating. In fact, if a 150-fs laser pulse with an average energy of 3.75 nJ is used, one can expect intensities in the evanescent zone of the waveguide between $0.1\,\mathrm{GW/cm^2}$ and $1\,\mathrm{GW/cm^2}$, assuming an effective waveguide cross section of $7.5 \times 10^{-7}\,\mathrm{cm^2}$ ($150\,\mathrm{nm} \cdot 0.5\,\mathrm{mm}$) and 10% coupling efficiency. This intensity range exceeds by orders of magnitude the lower limit of intensities ($0.1\,\mathrm{MW/cm^2}$ up to $1.0\,\mathrm{MW/cm^2}$) referred to in the literature [6] for observation of TPE fluorescence. The value of 10% coupling efficiency considers partial acceptance of the 7-nm bandwidth by the coupling grating. Additional pulse broadening may occur by group-velocity dispersion during passage of the short pulse through the waveguide.

4　　　Conclusions

We have demonstrated two-dimensional TPE of fluorescence in the evanescent field of metal oxide thin-film waveguides on macroscopic areas exceeding $20\,\mathrm{mm^2}$. This is several orders of magnitude larger than the excitation area in the usual configurations for TPE using focused beams from high-power lasers. The evidence of TPE was deduced from three indications: (i) the absence of fluorescence in regions without rhodamine B; (ii) a typical rhodamine B fluorescence spectrum observed by the eye; and (iii) the clear second-order dependence of the fluorescence intensity on the excitation intensity. This opens the perspective that TPE of fluorescence, so far limited to microscopic studies, can be established as a new spectroscopic method with higher sensitivity for macroscopic photophysical or photochemical studies of materials or components.

In future it can be expected that the detection limits can still be improved considerably upon optimisation of the experimental conditions for implementation of two-photon fluorescence excitation on extended planar waveguide surfaces. If this technique is applied to chemical and biochemical sensors based on planar waveguides, for which a superior sensitivity has already been demonstrated for configurations based on regular fluorescence excitation in the evanescent field [5], and which have found application as transducers for genomic and proteomic microarrays [8], we expect background-free fluorescence signals without any disturbing stray light from the bulk medium even upon the use of laser pulses of moderate peak power and picosecond duration.

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