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# Two-photon fluorescence excitation of macroscopic areas on planar waveguides☆

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**Abstract**

In this paper, we report the first successful demonstration, to our knowledge, of two-photon fluorescence excitation (TPFE) using planar thin-film waveguide structures of macroscopic excitation dimensions (square millimeters to square centimeters in size). The high intensity of excitation light required for TPFE is available not only at a single focus point but along the whole trace of the beam guided in the waveguide structure. Line profiles of the fluorescence excited by TPFE show excellent correlation with the geometry of the launched laser beams. A clear second-order dependence of the fluorescence intensity on the excitation intensity confirms the two-photon character of fluorescence generation. Spectra of the emission generated by one-photon excitation and by two-photon excitation show only minor differences.


*Keywords:* Two-photon excitation; Fluorescence; Planar waveguide; Microarray

## 1. Introduction

### 1.1. One- and two-photon excitation of fluorescence

The detection limits of fluorescence-based sensing configurations are most often not limited by the number of available fluorescence photons but by the background signals from insufficiently discriminated excitation light, especially in case of fluorophores with small spectral difference between the excitation and emission wavelengths. Consequently, larger spectral differences between the wavelengths of excitation and of the fluorescence to be detected are highly desirable but can most often only be achieved at the expense of lower absorption cross-sections or lower fluorescence quantum yields.

A powerful approach for overcoming this problem of spectral separation, i.e., for distinguishing a faint fluorescence signal from the background excitation noise, is provided by the concept of two-photon excitation (TPE) of fluorescence. TPE occurs, when a molecule is promoted by simultaneous absorption of two photons into an excited state, thereby passing through a virtual state. The excitation wavelengths, typically in the red or near infra-red, are selected in such a way that a molecular excitation cannot be achieved by absorption of a single photon (one-photon excitation, OPE) at the irradiated wavelength. As a prerequisite for successful TPE of a molecule, the simultaneously absorbed photons must have at least half of the energy required for excitation into the first excited state: whereas the emission spectra resulting from OPE or TPE are generally identical, the TPE peak wavelengths of many fluorophores appear blue-shifted with respect to twice of the OPE peak wavelengths. A red-shift

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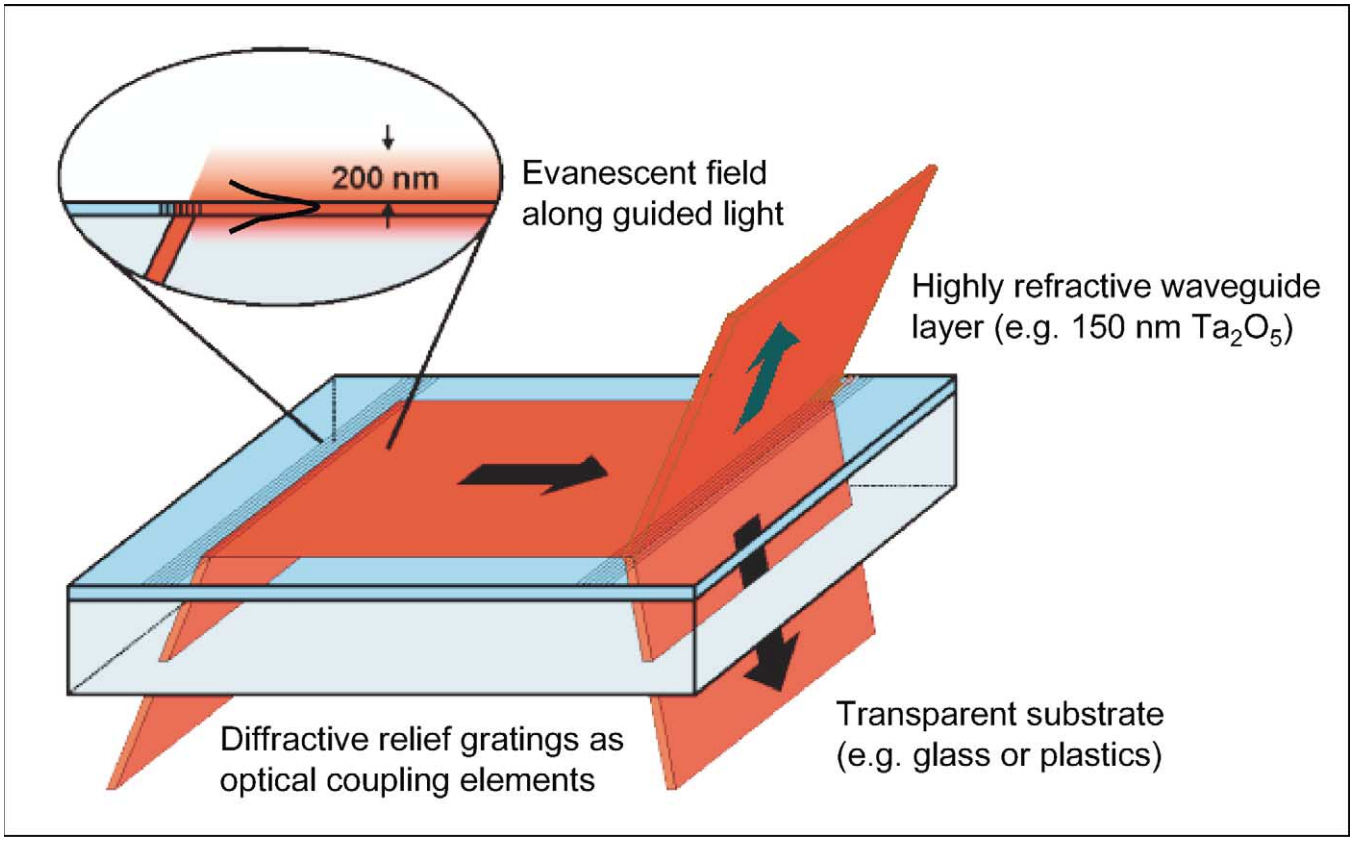


Fig. 1. Principle design of a thin-film waveguide structure for highly sensitive, surface-confined analyte detection.

relative to the OPE wavelengths has never been reported (Xu and Webb, 1996).

Due to TPE the emitted fluorescence and the excitation light are separated by a large energy gap. Direct excitation of any emission at still longer wavelengths than the launched excitation light by OPE is highly unlikely as only very few molecules can be excited to emit by the red or near-infrared excitation light. In addition, the spectral discrimination of any possible weak emissions in the red together with the intense but low-energetic excitation light against the short-wavelength fluorescence resulting from TPE is facilitated by the large energy gap. This discrimination can be further improved by proper choice of the detector since significant decrease in sensitivity at long wavelengths is inherent to many detectors. As a consequence, an improvement of signal-to-background and signal-to-noise ratios, compared to configurations based on regular OPE, can be achieved in spite of the well-known low quantum efficiencies of fluorescence generated by TPE.

However, high photon densities in excess of at least $10^5$ W/cm$^2$ are required for TPE (Amorim and Baravian, 2001). Therefore TPE had so far been performed only on excitation areas of the size of diffraction-limited focus of powerful lasers and the major application area currently is confocal microscopy in biological studies (Denk et al., 1990; Potter et al., 1996; Sako et al., 1997; So et al., 1998). First commercial systems for this application have only recently appeared on the market (Leica TCS MP Two Photon Imaging System).

### 1.2. Thin-film planar waveguides

High refractive index thin-film planar waveguides have been introduced as sensitive transducers for fluorescence-based (bio)sensors in the last decade (Duveneck et al., 1997; Rowe et al., 1999; Plowman et al., 1999) leading to an improvement of detection sensitivities on macroscopic sensor areas by about two orders of magnitude when compared to fiberoptic sensing configurations or to confocal scanning systems applying fluorescence generation by OPE (Ehrat and Kresbach, 2001).

This increase in sensitivity is enabled by the phenomena of evanescent field that is associated with light guiding in an optical waveguide. The evanescent field whose intensity decays exponentially with distance from the waveguide surface provides a tool for studies of molecular interactions confined to distances of macromolecular dimensions (i.e. some hundred nanometers depending on the irradiated excitation wavelength) from the waveguide surface. For these applications, biological or biochemical or synthetic recognition elements, for

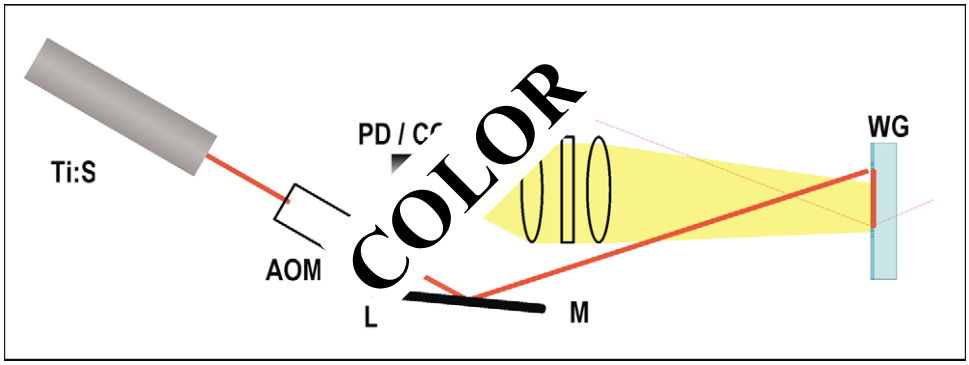


Fig. 2. Experimental setup for TPE on planar waveguides.

specific recognition and binding of the analytes to be detected, are immobilized on the waveguide surface.

The amount of energy available in the evanescent field increases with decreasing thickness of the waveguiding layer. This is true as long as the thickness is not reduced below the cut-off value for the actual excitation wavelength and direction of polarization, below which a guided wave can no longer be supported (Kunz et al., 1994). The evanescent field strength at the surface also increases with increasing difference between the refractive indices of the waveguiding layer and the adjacent media (e.g. a glass substrate and the sample solution). In practice, optimum results have been obtained with a thickness of 100–200 nm and a refractive index of 2 or higher (Fig. 1) (Duveneck, 1999). Under these conditions, 10% and even more of the guided light intensity can be confined to an interaction zone extending only about 200 nm from the waveguide surface. This high excitation intensity, which is comparable to the focal energy density in confocal microscopy when similar laser light sources are used, is not only available in a microscopic spot but along the whole path of the guided light wave. Thus, as a significant advantage compared to confocal scanners, no scanning is required for monitoring on macroscopic surfaces events of analyte binding to the immobilized recognition elements which is indicated by the fluorescence from labels attached to the analyte or one of its binding partners. Light in coupling into the waveguiding layer is with diffractive relief gratings (Fig. 1). Using optimized dimensioning of the coupling grating, more than 30% of the launched, monochromatic and parallel excitation light can be coupled into the waveguiding layer at resonance conditions.

We present results of first experiments showing the combination of TPE of fluorescence with thin-film planar waveguides which enables simultaneous TPE on surfaces as large as several square millimeters.

## 2. Experimental

### 2.1. Waveguide chip

As a waveguide structure (Unaxis Balzers, Liechtenstein), a thin-film waveguide was used with a 150 nm thin layer of $Ta_2O_5$ (refractive index $n = 2.092$ at 800 nm) on an AF45 glass substrate ($n = 1.496$ at 800 nm). Coupling of the excitation light from free space into the waveguide was obtained by using relief gratings with a period of 360 nm and a depth of 12 nm. These gratings (*length:* 0.5 mm) were provided at intervals of 9 mm on the waveguide structure (*length:* 48 mm, *width:* 16 mm), over its whole width. For a first series of experiments, a drop ($\approx 1$ μl) of Rhodamine B solution in ethanol ($1.6 \times 10^{-5}$ M for the experiment demonstrating first evidence of TPE, $5 \times 10^{-8}$ M for the later measurements) was deposited on the surface of the $Ta_2O_5$ layer between two gratings. After evaporation of the solvent, the Rhodamine molecules remained immobilized on the waveguiding layer, covering about a half of the available area between the gratings.

### 2.2. Optical system

A mode-locked titanium:sapphire laser ('Ti:S', Tsunami Model 3960, Spectra Physics, Mt. View, CA) operated at a frequency of 80 MHz was used as the excitation light source (Fig. 2). The emission of the laser was generally centered at 800 nm (if not indicated otherwise); the pulse width was 100 fs resulting in a spectral bandwidth of about 8 nm. The excitation light

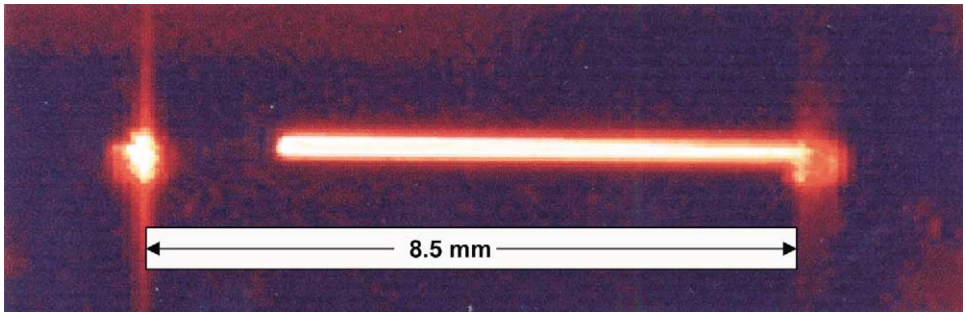


Fig. 3. Fluorescence from immobilized Rhodamine B generated by TPE, as it is observed by naked eye. The excitation light (800 nm) is coupled into the waveguiding layer at the grating on the left (bright spot) from where it is guided towards the right until the next, outcoupling grating. The path of the excitation light is invisible, until it reaches the region of the immobilized dye molecules.

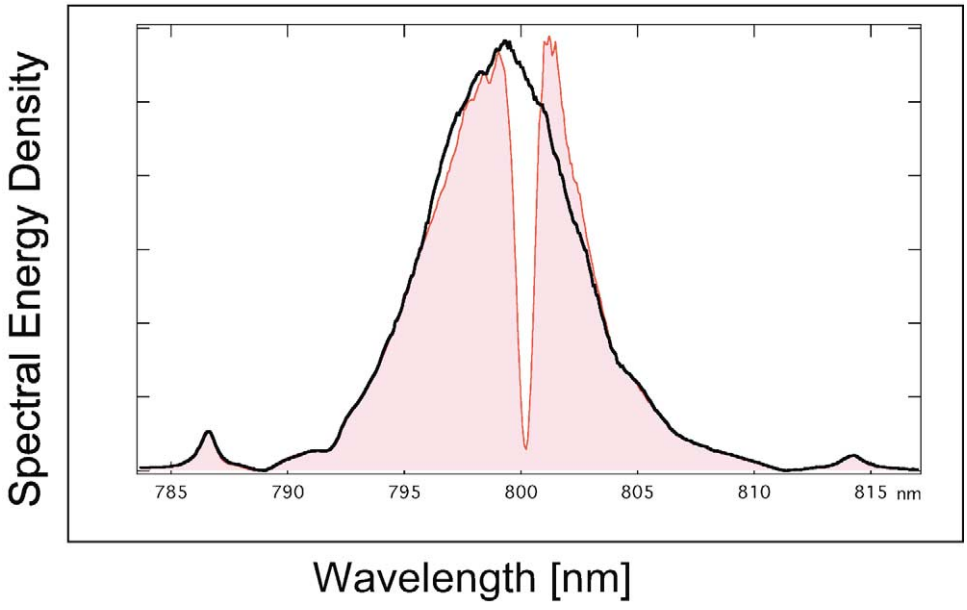


Fig. 4. Spectral selectivity of the coupling step. The irradiated excitation light has a spectral halfwidth of about 8 nm. For the adjusted angle, the resonance condition for light coupling into the grating waveguide structure is satisfied at 800 nm, where the spectrum of the transmitted light shows a sharp minimum.

was passed through an acousto-optical modulator ('AOM', AOM 602A11, Acousto-Optics, IntraAction Corp., Bellwood, IL) to allow continuous adjustment of the transmitted light and for chopping the light beam for lock-in detection of the collected emission signals. Exchangeable lenses ('L') were used in the excitation path to control the beam shape on the incoupling grating, where the excitation light was directed by a mirror ('M'). The beam waist of the launched excitation light on the incoupling grating could be reduced from about 400 μm (without lens) to about 100 μm (with a focusing lens) or expanded to several millimeters using a cylindrical lens. The waveguide chip ('WG') was mounted on a positioning stage allowing translation in two orthogonal directions (in parallel and perpendicular to the grating lines) and rotation around the axis of the incoupling grating placed in the beam waist so that a plane wave was incident upon it. At this position, the maximum incident average laser power was 600 mW, equivalent to about 7.5 nJ pulse energy with 44 kW peak power.

The emitted light was collected by a lens ('Obj') and imaged either on a camera ('CCD', AstroCam TE3/A, Cambridge, GB) for laterally resolved detection or focused onto a photodiode ('PD'), to detect a single spatially integrated signal. Optical filters could be placed in the emission light path to spectrally select transmission of the collected emission. For recording the spectrum of the amount of excitation light that passes the grating waveguide structure undiffracted, the transmitted light (at the opposite side of the waveguide chip with respect to the excitation light) was coupled into an optical fiber and observed with an optical spectrum analyzer (Advantest Q8381, Advantest Corp., Tokyo,

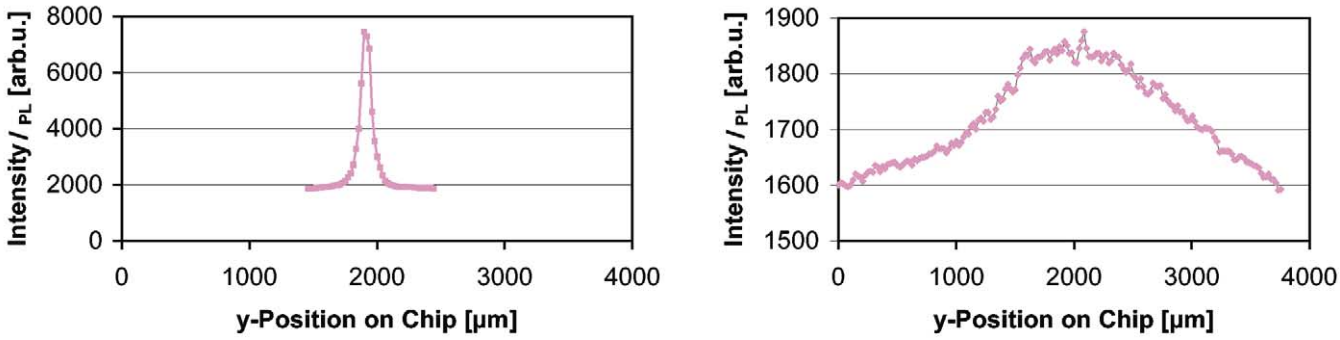


Fig. 5. Line profiles of the fluorescence generated by TPE for different geometries of the launched laser beam, taken perpendicular to the trace of the guided mode (in parallel to the grating lines, '$y$-position on chip'). *Left:* half-width of laser beam about 100 µm; *right:* half-width of laser beam about 2.5 mm.

Japan). For establishing spectra of the fluorescence generated by OPE or TPE, the fluorescence emitted isotropically along the path of the guided excitation light in the waveguide was collected by a lens located at the transmission side of the grating waveguide structure and focused onto the entrance slit of a miniature spectrometer with a CCD chip at its back side (S2000 Fiber Optic Spectrometer, Ocean Optics, Eerbeck, Netherlands). Saturation of the CCD chip by the intense excitation light from the titanium:sapphire laser was avoided by placing an NIR-blocking filter into the emission light path.

## 3. Results and discussion

### 3.1. First visual evidence for fluorescence generation by TPE

In a first experiment, the laser-beam (500 mW) was slightly focused to a 100 µm beam waist and aligned at the coupling resonance angle on the grating (left hand side of Fig. 3). The resulting emission from the waveguide structure could be observed by the naked eye and recorded with a commercial digital camera (Nikon Coolpix 990) without a filter in front of the camera. The coupling position on the grating (at a resonance angle of $-31^{\circ}$, i.e., in backward direction with respect of the direction of propagation of the guided mode) is evident from the bright light spot, from where the laser field propagates in the waveguiding layer towards the next grating (right hand side of Fig. 3) where it is finally coupled out. The first part of propagation of the guided 800 nm excitation light in the waveguide film without Rhodamine B coverage is invisible since the intensity of the much weaker scattered light associated with the guided mode in the unstructured parts of the grating waveguide structure is well below the detection limit of the camera for this spectral region. Starting from the point where the Rhodamine B solution has been deposited, a strong emission is observed with the typical yellow color of the Rhodamine B fluorescence. The outcoupling position at the next grating to the right is evident from the disappearance of the emission signal. The absence of any observable light signal along the propagation path where there is no dye is a first clear indication that nonlinear process other than TPE like surface-confined second harmonic generation can be excluded as causes of the observed emission.

### 3.2. Spectral selectivity of the coupling step

Fig. 4 shows the spectral energy density observed in direction of the transmitted light when the excitation

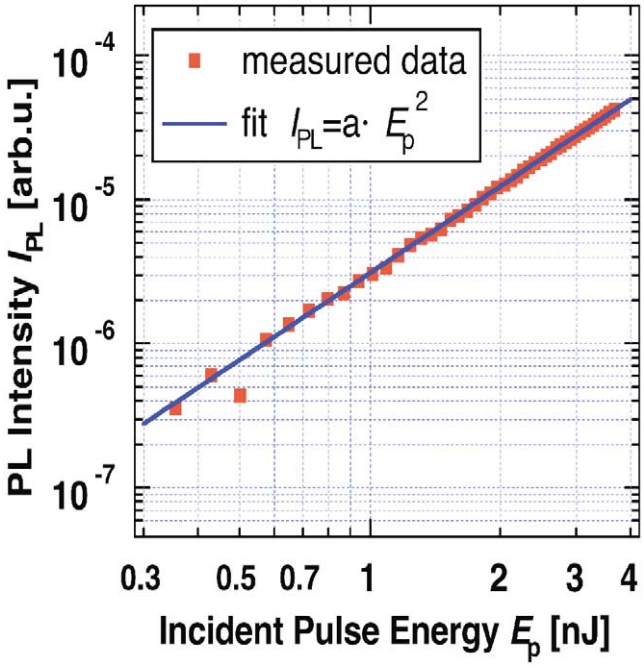


Fig. 6. Fluorescence intensity as a function of the incident pulse energy $E_P$: pure quadratic dependence without any linear or higher order component-clear evidence for a second-order excitation process (TPE).

light was launched under coupling conditions approximately onto the middle of the coupling grating (which is different from the position for optimum incoupling of light into the unstructured part of the waveguiding layer, located at the edge of the grating in direction of propagation of guided light). Under the adjusted conditions, the resonant launching angle for light coupling into the waveguiding film is satisfied at 800 nm. At this wavelength, a very sharp decrease of the amount of transmitted light is observed in the spectrum of the excitation light. The halfwidth of this resonance is about 1 nm, compared to about 8 nm halfwidth of the irradiating excitation light spectrum. The decrease of the transmission approaches almost complete extinction of the transmitted light for the resonance wavelength at this position on the grating.

The sharp spectral selection of light coupling demonstrated in this experiment explains the relatively low coupling efficiency ( $\sim 4\%$) observed in this configuration using 100 fs short laser pulses. The bandwidth of 1 nm of the excitation light accepted by the coupling grating corresponds to a minimum (bandwidth-limited) pulse duration of 700 fs according to the Fourier transformation. It has to be concluded that shorter pulses will only result in decreased efficiency of the coupling process and that laser pulses of 1 ps or longer duration should be applied for optimum light coupling.

### 3.3. Degree of agreement between excitation and emission profiles

In another experiment, the degree of agreement between the shape of the launched excitation light spot on the incoupling grating and the profile of generated emission, as well as the ability to generate fluorescence, presumably by TPE, simultaneously on extended areas of the waveguide chip were investigated. Lenses with different focusing properties were inserted in the excitation light path (according to Fig. 2) and the resulting emission along the traces of the guided mode recorded by a sensitive CCD camera. An interference filter with maximum transmission at the peak of the Rhodamine fluorescence ( $\sim 580$ nm) was placed in front of the camera. One-dimensional profiles of the recorded emission, taken from the digitized laterally resolved camera images in parallel to the grating lines and perpendicular to the trace of the guided mode are shown in Fig. 5. Beam diameters of approximately 0.1, 0.4, and 2.5 mm were generated on the coupling grating in parallel to the grating lines and the resulting emission profiles showed perfect agreement with these geometries of the excitation light traces. This is demonstrated on the example of emission profiles resulting from 0.1 to 2.5 mm excitation beam halfwidth (Fig. 5).

### 3.4. Dependence of the emission intensity on the excitation light intensity

In order to clearly identify the physical nature of the excitation process for the observed Rhodamine B emission, the excitation light intensity was increased stepwise from 0 to 300 mW average power using the AOM. The resulting integrated emission intensity was measured using a GaAsP photodiode. The excitation light was chopped (2 kHz) and the signals from the photodiode were detected with a lock-in amplifier to suppress environmental light signals. Fig. 6 shows a double-logarithmic presentation of the recorded emission intensity as a function of the excitation light intensity, confirming a perfect quadratic fit of the experimental data without any contribution from linear or higher order components. This result demonstrates clearly together with the evidences presented above that the observed fluorescence is caused by TPE.

### 3.5. Spectra of fluorescence generated by OPE and by TPE

Spectra of the Rhodamine B fluorescence on the same waveguide chip, generated by regular OPE by means of a small frequency-doubled Nd:$YVO_4$ laser (532 nm) and by TPE using the titanium:sapphire laser adjusted to 814 nm, are displayed in Fig. 7. The spectra of the emission generated by the two different excitation processes are in good agreement with a slight shift of the emission maximum towards longer wavelength after TPE. In the upper spectrum of the fluorescence generated by OPE, the spectrometer is saturated at the wavelength of the excitation light (532 nm). In the lower curve of the emission generated by TPE, the NIR excitation wavelength is evident from the signal peak at 814 nm, well separated from the fluorescence band. It has to be pointed out that the fluorescence spectrum generated by TPE shows an excellent quality indicating a similar signal-to-noise ratio as the spectrum generated by OPE, under conditions that are still far away from optimization. The fact that no emission was detected in the spectral region between 350 and 500 nm additionally allows to exclude any other nonlinear effect other than TPE (e.g. second harmonic generation).

## 4. Conclusions

Simultaneous TPE of fluorescence has been demonstrated for the first time on extended areas on planar thin-film waveguides. The observed emission traces showed perfect agreement with the excitation beam profiles launched into the incoupling grating, and excitation areas could be extended to more than 20 $mm^2$. The unambiguous identification of the physical

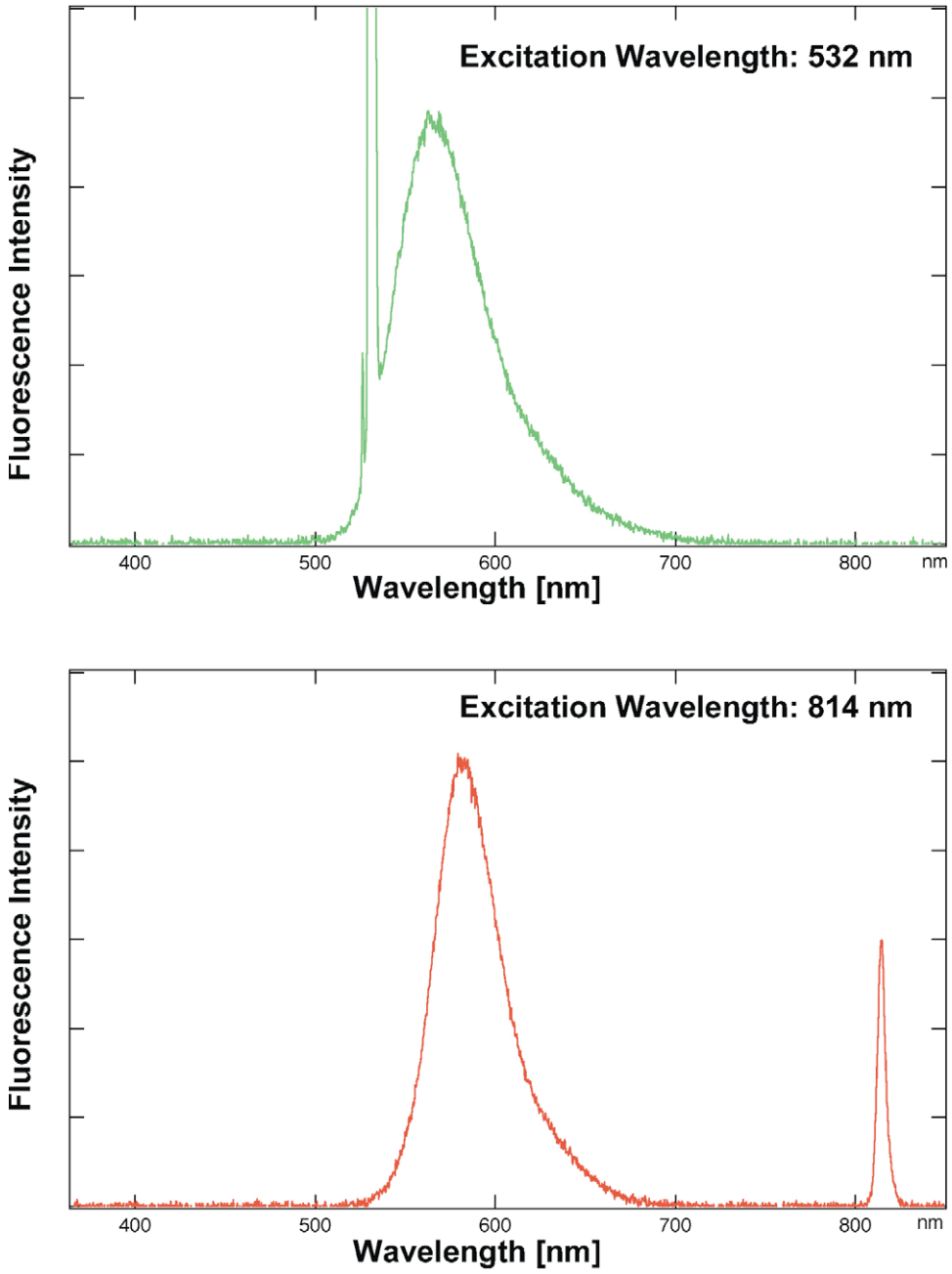


Fig. 7. Fluorescence spectra of Rhodamine B after OPE at 532 nm (upper curve) and TPE at 814 nm (bottom curve). The concentration of the deposited Rhodamine B sample (1 μl) was $5 \times 10^{-8}$ M in ethanol.

nature of the excitation process as TPE is based on the absence of any emission in regions without the immobilized dye, thus allowing to exclude any other nonlinear processes as excitation pathways. The spectral agreement between the emission observed in these experiments and the well-known fluorescence spectrum of Rhodamine B generated by OPE and, last but not least, the perfect quadratic dependence of the observed emission intensity on the excitation light intensity confirms TPE. The fluorescence spectrum resulting from TPE additionally allows to exclude any effects of second harmonic generation.

The drastic increase of the TPE area, by up to six orders of magnitude in comparison to known configurations in confocal microscopy, has been explained by the confinement of the whole intensity of a guided wave

and its associated evanescent field in the adjacent media to sub-wavelength dimensions, in the direction perpendicular to its propagation in the waveguide, whereas this high excitation intensity is simultaneously available along the whole area traversed by the guided wave in its plane of propagation. The importances of the adaptation of the excitation light properties, especially its spectral bandwidth and of the excitation light pulse duration on the coupling efficiency have been discussed.

Based on our results, it can be envisioned that the new combination of planar waveguide technology with TPE will become a powerful tool for macroscopic surface interaction studies. In the future TPE will lead to a further improvement of the sensitivity of biosensing platforms like genomic and proteomic microarrays based upon planar waveguides.

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