

Two-photon fluorescence: large area excitation and enhanced sensitivity using waveguide structures

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Using the evanescent field of thin-film waveguides, we demonstrate the possibility to induce TPF excitation simultaneously on large areas. Also, the application of diffractive optics for parallel excitation of TPF is investigated. These concepts are then applied to the excitation of the intrinsic fluorescence of amino acids.

1 Introduction

Two-photon fluorescence (TPF) microscopy is characterized by high resolution, three-dimensional imaging capabilities, large penetration depth and reduced photobleaching. It especially qualifies for studies of tissue and living cells. Furthermore, biochemical labeling becomes dispensable in some applications.

On the other hand, TPF excitation requires high intensities only available in the focus of ultra-short-pulse lasers. This restriction to very small excitation areas is a major drawback in pharmaceutical screening systems, where a large number of samples have to be analyzed in parallel.

2 Waveguide excitation

2.1 Planar waveguides

As an approach for the parallel excitation of TPF on large areas, we use the strong evanescent field of planar thin-film waveguides. The sample resides in close proximity to the waveguide surface and is illuminated by the evanescent field of the guided mode (Fig. 1). Confined to a layer of typically a few hundred nm thickness, the intensity of the evanescent field equals that of a focused laser beam. Yet, in contrast to the small focal spot, this high intensity is achieved over the whole surface of the waveguide. Using planar waveguides in combination with different dyes, two-photon excitation on areas of several mm² could be realized, thus exceeding the excited area in the diffraction limited laser focus by up to eight orders of magnitude [1].

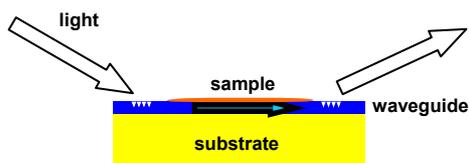


Fig. 1 Principle design of a thin-film waveguide structure.

2.2 Grating waveguide structures (GWS)

A strong TPF signal enhancement can also be achieved with grating waveguide structures (GWS). These are thin-film waveguides which are continuously structured with a grating between waveguide and substrate and/or on top of the waveguide (Fig. 2). Under resonance conditions, i.e. specific wavelength, polarization and angle of incidence, these structures support a strong guided mode and show vanishing transmission and maximum reflection.

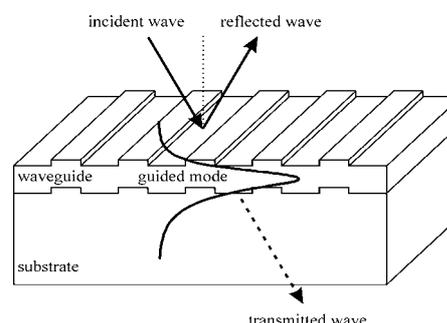


Fig. 2 Schematic illustration of a double grating waveguide structure (DGWS).

In theory, GWS can be described by a multiple interference model and interpreted as inverted Fabry-Perot interferometers. The resonance conditions depend on layer thickness, index of refraction and on grating period and depth. The spectral and angular acceptance, i.e. the bandwidth of the resonance, is larger for double grating waveguide structures (DGWS) compared to structures with a single grating [2].

Again, the evanescent field of the guided mode can be used for TPF excitation of surface confined dye molecules. Compared to planar waveguides, GWS allow the coupling of the laser source at the location of the sample, thus facilitating a much simpler illumination system. A typical experimental setup is illustrated in Fig. 3.

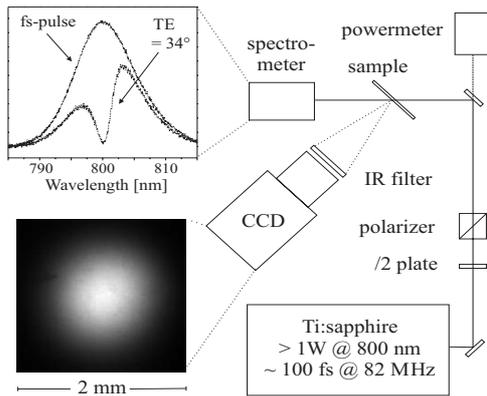


Fig. 3 Experimental setup including spectra of the fs-pulses with sample in resonance and without sample (top left) as well as camera image of two-photon fluorescence taken under resonance condition (bottom left).

Fig. 4 shows the TPF signal versus angular orientation of the incident light that was obtained from Rhodamine B on a DGWS sample with a concentration of 1.6×10^{-12} mol/cm². The DGWS consists of a 150 nm thick film of Ta₂O₅ on a glass substrate and a grating with a period of 360 nm and a depth of 40 nm. The signal enhancement under resonance conditions is clearly visible. A thorough analysis reveals a 350-fold enhancement compared to direct, non-resonant excitation [3]. The angular acceptance amounts to a few degrees. A similar but less pronounced enhancement can also be observed using the amino acid tryptophan instead of Rhodamin B.

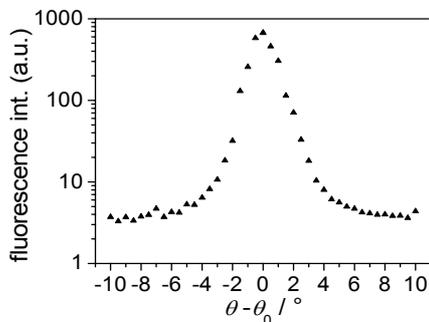


Fig. 4 Dependence of the TPF signal from Rhodamine B on a double grating waveguide structure on the angle of incidence.

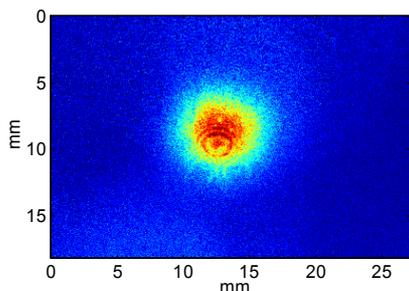


Fig. 5 TPF signal from Rhodamin B on a DGWS substrate in color coding. The laser beam was expanded to a spot size of ~10 mm diameter.

The possibility to achieve large area TPF excitation on structured waveguides is demonstrated in Fig. 5. In the associated experiment, the laser beam was expanded to a diameter of ~10 mm. A strong TPF signal could be recorded over the whole cross-sectional area of the laser beam of ~75 mm². This figure must be compared to the typical excitation area of a few μm² in regular two-photon microscopy.

3 Parallel excitation with Diffractive Optical Elements

To realize the simultaneous excitation of TPF on an array of discrete spots, the laser beam can be split into a number of separate beams by means of a diffractive optical element (DOE). Fig. 6 shows the fluorescence recording when exciting tryptophan on a glass substrate in a 3x3 focal array. The employed four-step diffractive phase element was made from polycarbonate by direct laser ablation at 193 nm [4]. A distinct TPF signal can be observed in all focal spots, albeit with strong intensity variations between the single spots. Note however, that existing variations in the incident intensity are amplified by the quadratic dependence of the TPF signal.

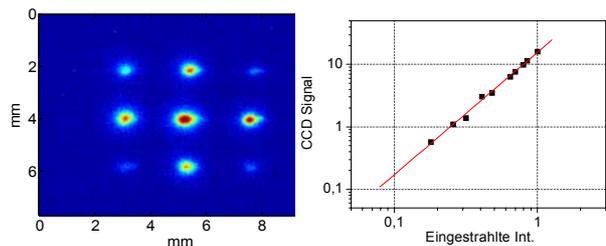


Fig. 6 TPF signal from Tryptophan on glass substrate in a 3x3 focal array generated by a diffractive phase element in color coding. Left: CCD signal. Right: the second order dependence on the incident intensity proves the pure two-photon excitation.

References

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