

# **Nonlinear delayed fluorescence in confocal scanning microscopy. Point-spread-function analysis**

MAREK KOWALCZYK

Institute of Geophysics, Warsaw University, ul. Pasteura 7, 02–093 Warszawa, Poland.

MANUEL MARTÍNEZ-CORRAL, CARLOS J. ZAPATA RODRÍGUEZ

Departamento de Optica, Universidad de Valencia, Dr. Moliner 50, 46100 Burjassot, Spain.

Generation of the signal beam in the confocal scanning fluorescence microscopy by means of nonlinear delayed fluorescence phenomenon based on triplet-triplet annihilation is proposed. From the point of view of the spatial resolution of three-dimensional confocal imaging, the superiority of the phenomenon proposed here over the classical two-photon fluorescence is demonstrated. This is done in terms of axial and lateral point spread functions computed for the assumed model of nonlinearity.

## **1. Introduction**

The two-photon (2-p) excited fluorescence, one of the most important recent developments in confocal fluorescence microscopy, improved the quality of three-dimensional (3-D) microscopic images [1]. It is a usual practice to relate this good quality with an improved spatial resolution which, in turn, could be attributed to nonlinear dependence of the fluorescence signal on excitation intensity. Nevertheless, there is another factor, not necessarily secondary, which improves 3-D microscopic images obtained by means of 2-p technique. This is the good penetration of biological tissues by infrared radiation, which is frequently used for 2-p excitation, whereas the green or blue light which is used to excite one-photon (1-p) fluorescence is highly absorbed in tissues.

However, there are also some drawbacks of 2-p confocal fluorescence imaging. As a result of small cross-section for the 2-p absorption on the one hand, and the requirement of a fast image acquisition, on the other, high-power impulse lasers have to be used. This affects the specimen under investigation and the fluorescence dye itself (photobleaching). Therefore, nonlinear fluorescence phenomena, other than those excited by means of the classical 2-p absorption via a virtual state of femtosecond lifetime, should be considered. In this line HÄNNINEN *et al.* [2] proposed a conjugate dyes system (donor and acceptor molecules) in which prompt fluorescence of the donor molecule excited by a single photon is observed. The role of the second photon is to excite the acceptor molecule and in this way inhibit the donor-acceptor energy transfer, which could be followed by a prompt fluorescence

of the acceptor or nonradiative dissipation of its energy. SCHÖNLE *et al.* [3] extended this concept to highly nonlinear multiphoton cascaded processes. Another possibility is offered by trichromophoric compounds in which two chromophoric groups play the role of donors (antennas). The first excited singlet state of the acceptor chromophoric group (target) is such that it can be populated only by means of virtually simultaneous intramolecular energy transfer from excited donors [4], [5]. In this way a 2-p excitation is involved.

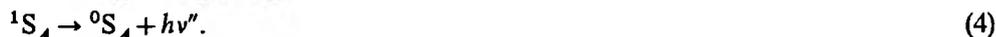
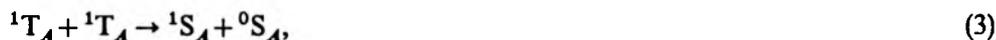
In this paper, we propose the use of the pyrene-type sensitized delayed fluorescence (PSDF) as the mechanism which generates the signal beam in the scanning confocal fluorescence microscopy. We consider here the sensitized process and not the direct one as the former is much more efficient. The nonlinear dependence of the PSDF versus exciting intensity was revealed by PARKER [6].

## 2. Description of the process

The PSDF assumes an energy transfer between an excited donor molecule D in its first triplet state  ${}^1T_D$  and an acceptor molecule A in its ground singlet state  ${}^0S_A$ :



Then the self-quenching of triplet acceptors (triplet-triplet annihilation) followed by fluorescence of the excited monomer acceptor takes place:



Optical frequencies  $\nu'$  and  $\nu''$  correspond to the exciting and fluorescence light beams, respectively. We have here a kind of the 2-p process as two photons are needed to produce two triplet acceptors whose annihilation yields a single photon  $h\nu''$ .

If the energy levels are matched in such a way that the energy of  ${}^1T_D$  is higher than that of  ${}^1T_A$  and the energy of  ${}^1S_D$  is smaller than that of  ${}^1S_A$ , then the

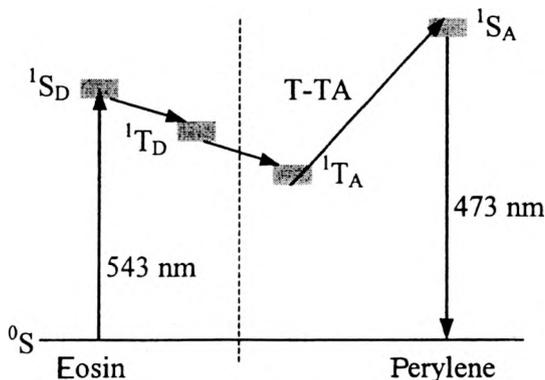


Fig. 1. The Jablonski diagram for eosin and perylene; T-TA — excitation via triplet-triplet annihilation.

frequency  $\nu'' > \nu'$ . Examples of the donor-acceptor pairs that meet such conditions include: fluoranthene – pyrene, proflavine – pyrene, phenantrene – naphthalene, proflavine – 3,4 benzpyrene, eosin – perylene (Fig. 1). From among the dyes mentioned above it is especially perylene that is used in biomedical fluorescence imaging. It stains membranes and in particular their hydrophobic parts [7].

As the PSDF is a 2-p process it demonstrates a quadratic dependence between the fluorescence and exciting intensities. However, the above is true only for low excitation levels. For higher intensities a linear relation is observed [6]. In the section to follow we analyse in detail how this affects the spatial resolution of 3-D confocal imaging and compare the results with those for a standard 1-p and 2-p confocal fluorescence microscopy.

### 3. Lateral and axial point spread functions

In our simulations we apply the following assumptions:

– The nonlinear dependence between normalized excitation and emission intensities is modelled with the use of a truncated Huber function [8]

$$N(s; T) = \frac{1}{2T - T^2} \begin{cases} s^2, & 0 \leq s < T, \\ T^2 + 2T(s - T), & T \leq s \leq 1, \\ 0 & \text{otherwise.} \end{cases} \quad (5)$$

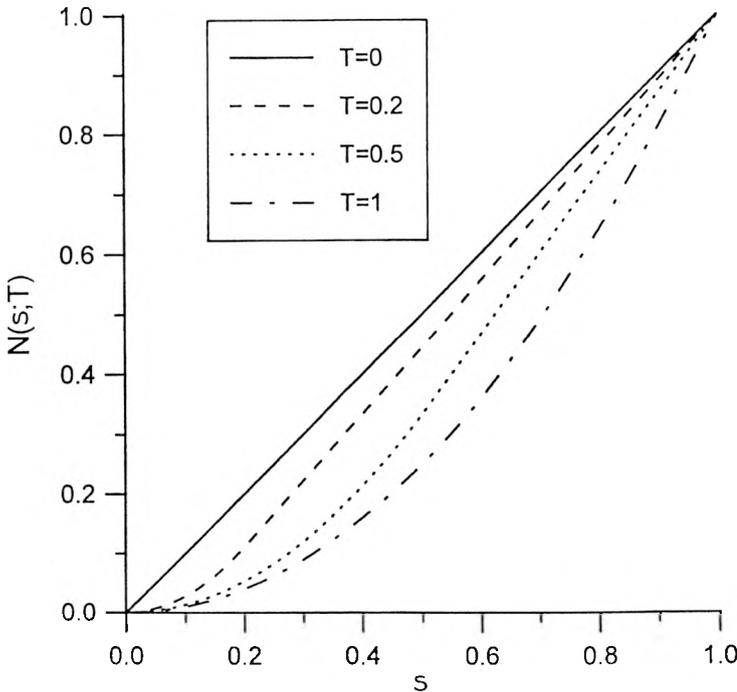


Fig. 2. Graph of the Huber functions;  $T$  – coordinate of the transition point that separates intervals of parabolic and linear behaviour.

This function is parabolic for  $s < T$ . It softly transforms into linear function when its argument increases (Fig. 2).

- All of the optical elements are axially symmetric.
- The 3-D point spread function  $h_i$  of the confocal imaging is given by [1]

$$h_i(r, z) = N[h_1^2(r, z)] h_2^2(r, z) \quad (6)$$

where  $h_1$  and  $h_2$  are the amplitude impulse responses of the illuminating and collecting parts of the system, respectively;  $r$  and  $z$  are radial and axial cylindrical coordinates such that  $z = 0$  corresponds to the confocal plane, and the transformation  $N[h_1^2]$  is given by Eq. (5) for  $s = h_1^2$ .

- The pupil functions are circular (some results for microscopic imaging by means of 2-p confocal apodized system can be found in [9] and [10]).

- The system is free of aberrations and its numerical aperture is set to 0.75. For simplicity we use this value for all the wavelengths involved.

- The fluorescence dye is perylene dissolved in N,N-dimethylformamide, so that the wavelength of fluorescence is 473 nm.

- The excitation wavelengths  $\lambda_{ex}$  and sources are those summarized in the Table.

Table. Laser sources of excitation beam and corresponding wavelength for three different kinds of excitation.

One-photon	Two-photon	Sensitized T–T annihilation
Ar <sup>+</sup> (458 nm) N <sub>2</sub> (327 nm)	Ti:sapphire (946 nm)	Green He-Ne (543 nm)

- Eosin is used as sensitizer; it can be dissolved in N,N-dimethylformamide as well as perylene [11] and can be excited with green He-Ne laser (Tab.).

For the PSDF the lateral and axial cross-sections of the 3-D point spread functions  $h_i(r, z)$  are presented in Figs. 3 and 4, respectively, and are compared with those for standard 2-p excited fluorescence confocal microscopy in which a Ti:sapphire laser is used. They were calculated using routine Fourier-optics methods [12]. The results presented in Figs. 3 and 4 demonstrate theoretical advantage of PSDF over 2-p excited fluorescence. In fact, such a superiority of spatial resolution with respect to that of 2-p excited fluorescence takes place also for 1-p fluorescence. This is shown in Fig. 5. Thus, it is seen that when point spread functions are expressed in absolute units and true wavelengths are taken into account, the gain of resolution due to the nonlinear excitation is fully cancelled by increment of the volume in which the excitation takes place. This volume is proportional to  $\lambda_{ex}^3$ , which for infrared excitation is essentially larger than that for a blue one.

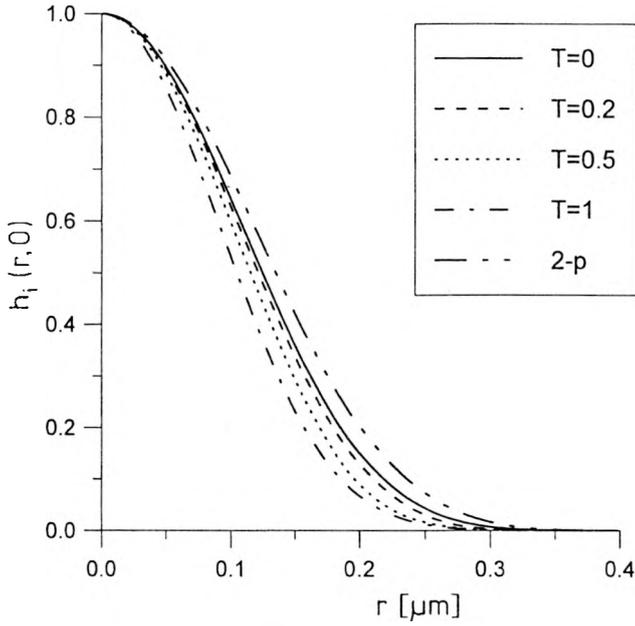


Fig. 3. Lateral cross-sections of 3-D point spread functions of the confocal microscope in which PSDF is employed. Normalized intensity of excitation  $h_1^2$  which is smaller than  $T$  assumes parabolic dependence between excitation and fluorescence intensities.

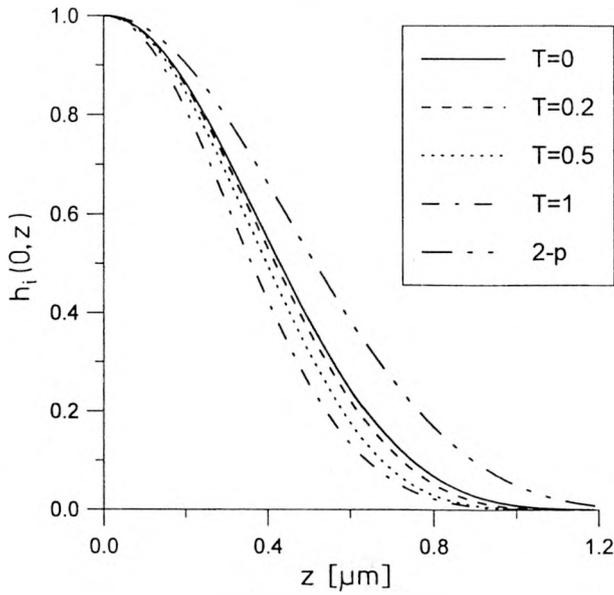


Fig. 4. Same as in Fig. 3, but for axial cross-sections.

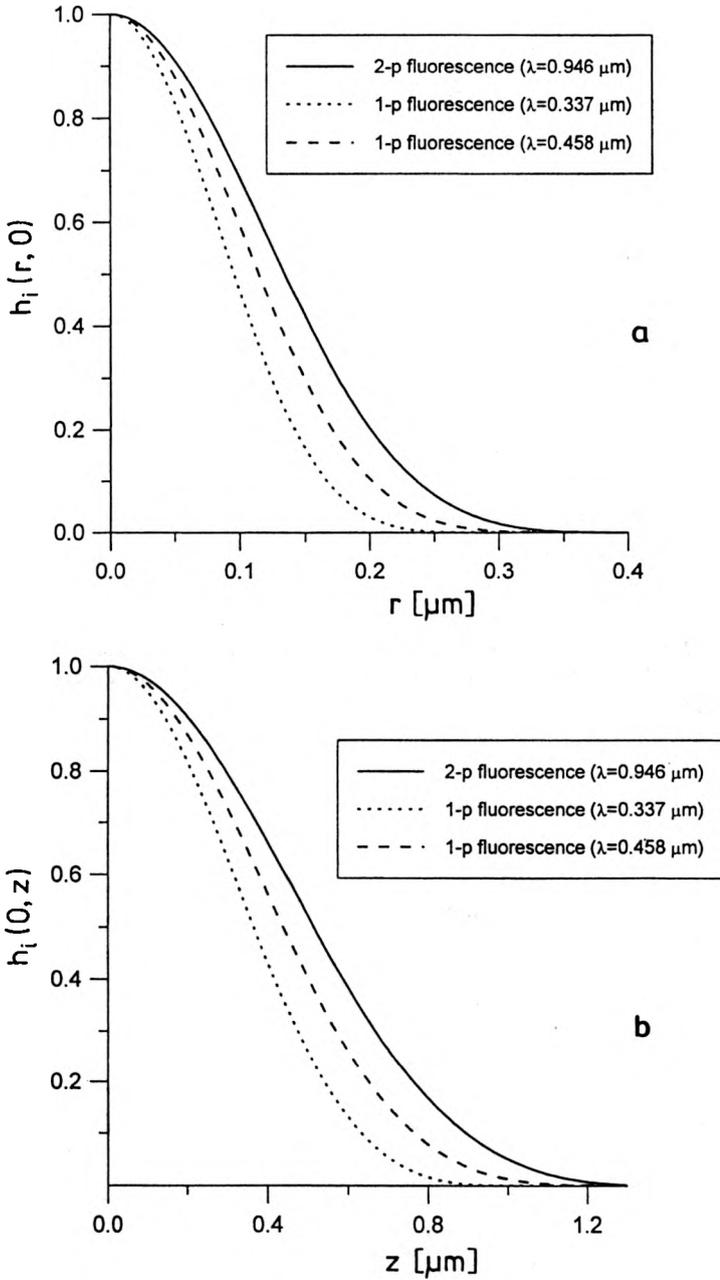


Fig. 5. Lateral (a) and axial (b) cross-sections of the 3-D point spread function in confocal scanning microscope in which either 1-p or 2-p excited fluorescence is employed. In both cases the wavelength of fluorescence is 473 nm.

## 4. Conclusions

Our simulation demonstrates that PSDF yields the point spread functions of fluorescence confocal imaging that are by 25% narrower than those for 2-p fluorescence (in terms of FWHM) and are of the same width as those for 1-p fluorescence. On the other hand, the latter takes place when the wavelengths of excitation are, for example, 543 nm for PSDF and about 400 nm for 1-p fluorescence. Therefore, from the point of view of absorption in tissues and stability of dye molecules, the PSDF is a better choice. In view of our results, good quality of 2-p fluorescence imaging is owed rather to high signal to noise ratio and not to an improved spatial resolution. If it were contrary, the extensively used quadratic relationship between excitation and fluorescence intensities would have to be refined towards step-like nonlinear dependence.

Among other factors, the long lifetimes of excited triplet states determine the kinetics of PSDF. This has two important consequences:

- It is not necessary to use high power laser pulses to depopulate virtual states of extremely short lifetimes, which is the case for 2-p fluorescence.

- Unlike the 2-p fluorescence, which is a prompt process, the PSDF is a delayed one. As such it has some advantages which were outlined by JOVIN *et al.* [13]. For example, a properly gated signal of delayed fluorescence can be considered free of autofluorescence component and also free of glare and light scattered within the microscope.

There are also some drawbacks of the PSDF. From the point of view of dipole–dipole interaction the energy transfer of Eq. (2) is spin forbidden, so it takes place only in encounters (Dexter model [14]) and as such it is less efficient than fluorescence resonance energy transfer (FRET) (Förster model [15]). Thus, at the stage of acquisition of the image data one should consider the application of the lock-in detection or even a photon counting. Another difficulty is a proper selection of donor and acceptor compounds. The diagram of energy levels (Fig. 1) is not a unique criterion. Both compounds should be soluble in the same solvent, which in turn should be “acceptable” for biological tissues. Also the quantum yields and lifetimes should favour the processes of Eqs. (1)–(4). In liquid media the PSDF assumes some diffusion of the species involved, thus the fluorophores in question can be used rather as physiological reporters and not labels.

It should be admitted that along with investigation of new nonlinear fluorescence phenomena a significant progress has been done in the field of fluorophores for standard 2-p microscopy: a new generation of fluorescent molecules with large cross-section for 2-p absorption has been developed [16]. Nevertheless, it seems that the future of multiphoton fluorescence microscopy is connected with low intensity beams of entangled photons, which are particularly well suited to excite the 2-p or three-photon fluorescence [17]–[19].

## References

- [1] GU M., *Principles of Three-Dimensional Imaging in Confocal Microscopes*, World Sci., Singapore 1996.
- [2] HÄNNINEN P.E., LEHTELA L., HELL S.W., *Optics Commun.* **130** (1996), 29.
- [3] SCHÖNLE A., HÄNNINEN P., HELL S.W., *Ann. Phys. (Leipzig)* **8** (1999), 115.
- [4] NICKOLEIT M., *et al.*, *J. Inform. Rec.* **23** (1996), 63.
- [5] NICKOLEIT M., UHL A., BENDIG J., *Laser Chem.* **17** (1997), 161.
- [6] PARKER C.A., *Photoluminescence of Solutions*, Elsevier, Amsterdam 1968.
- [7] LAKOWICZ J.R., *Principles of Fluorescence Spectroscopy*, Plenum Press, New York 1983.
- [8] HUBER P.J., *Robust Statistics*, Wiley, New York 1981.
- [9] MAGIERA A., *Atti Fond. Giorgio Ronchi* **50** (1995), 489.
- [10] MAGIERA A., *Opt. Appl.* **28** (1998), 249.
- [11] MAEDA M., *Laser Dyes*, Academic Press., Tokyo 1984.
- [12] KOWALCZYK M., ZAPATA-RODRIGUEZ C.J., MARTINEZ-CORRAL M., *Appl. Opt.* **37** (1998), 8206.
- [13] JOVIN T.M., *et al.*, *Ber. Bunsenges. Phys. Chem.* **93** (1989), 387.
- [14] DEXTER D.L., *J. Chem. Phys.* **21** (1953), 836.
- [15] FÖRSTER T., *Discuss. Faraday Soc.* **27** (1959), 7.
- [16] CHENG P.C., *et al.*, *J. Microsc. (Oxford)* **189** (1988), 199.
- [17] TEICH M.C., SALEH B.E. A., *Československý časopis pro fyziku* **47** (1997), 3.
- [18] TEICH M.C., SALEH B.E. A., *Entangled-Photon Microscopy, Spectroscopy, and Display*, US Patent 5 796 477, 1998.
- [19] BOUWMEESTER D., *et al.*, *Phys. Rev. Lett.* **82** (1999), 1345.

Received May 22, 2000