Investigation of transient two-photon excited fluorescence in biological tissue

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The fluorescence power from biological tissue excited by a femtosecond laser pulse compared with excitation power does not appear to obey a simple quadratic relationship given by the steady non-linear theory. A more reliable analysis is developed based on transient two-photon absorption because the response time of two-photon absorption is longer than the width of a femtosecond pulse. Good agreement is obtained between the theoretical analysis and the experimental results of fluorescence power versus excitation power. This letter offers potential value to non-linear optics in biological tissues.

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Since the introduction of two-photon scanning laser microscopy into the field of functional microscopic biology[1−5], non-linear effects—especially on two-photon excited fluorescence (TPEF)—have been widely applied to biological sciences. Applications have exhibited a number of advantages, including efficient background rejection, inherent optically sectioning, three-dimensional (3D) reconstruction, deep optical penetration, and minimal out-of-plane photo-bleaching[6]. Although TPEF has extensive applications in biological imaging, detection, and diagnosis, a limited number of groups have focused on basic non-linear theory and effect from light-matter interaction[7−9]. For example, what is the relationship of TPEF on the excitation power? This simple question has rarely been examined. Considering the fact that the response time of the two-photon absorption is longer than the width of a femtosecond pulse, it may not be accurate to claim a quadratic law of TPEF on the excitation power that is suitable with steady non-linear theory.

In this letter, our primary goal is to investigate TPEF in biological tissue using a femtosecond pulse based on transient two-photon absorption. As a result, a more reliable relationship of TPEF power versus excitation power is presented. We believe that the result given here is useful to further study non-linear effects in tissue optics.

As shown in Fig. 1, TPEF is a non-linear process in which a fluorescent molecule that nearly simultaneously absorbs energies of two of the same photons is excited to a excited state and then emits fluorescence, which has a longer wavelength than half of the excitation wavelength because some of the incoming energy is lost during the relaxation of the excited state.

Based on the steady-state theory of the two-photon absorption for the case of weak absorption, the output intensity of light exiting from the medium can be expressed by[10]

$$I_1(z) = I_{10} - I_{10}^2\omega_1\beta z, \tag{1}$$

where I_{10} is the initial intensity of laser field, ω_1 is the frequency of excitation laser, β is the coefficient of two-photon absorption, and z is the thickness of the medium. As a whole, $I_{10}^2\omega_1\beta z$ represents the intensity absorbed by the medium due to two-photon absorption. This law may not be applicable to the TPEF because the response time of the two-photon absorption is approximately $10^{-11} - 10^{-10}$ s[10], which is of approximately two orders of magnitude longer than pulse width of a femtosecond laser; thus, the TPEF process should be considered a transient process. In addition, since the life of fluorescence is longer than the response time of two-photon absorption, as a primary approximation, it is usually valid that the energy of TPEF is proportional to the upper state population. This approach differs from the quadratic law of TPEF of the incident power as predicted by the steady-state non-linear theory.

To describe the transient TPEF, we should generally use the coupled wave approach. In this approach, the dynamic equation for the biological tissue excitation takes into account the possible transient response of the medium. The slow varying amplitude approximation of the fields is valid. The optical dispersion caused by tissues is neglected because the propagation length of the pulse and the dispersion length of tissues far outweighs the thickness of the specimen[11]. Following a derivation similar to that of the Raman scattering[12,13], the set of dynamic coupled equations for TPEF can be written

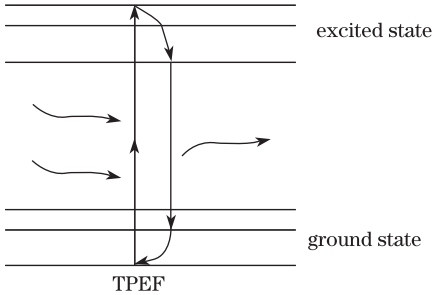

Fig. 1. Sketch of TPEF process.

as

$$\frac{\partial E_{\rm l}}{\partial z} = {\rm i}\kappa_{\rm l} Q_{\rm v} E_{\rm l}, \tag{2}$$

$$\frac{\partial Q_{\rm v}}{\partial t} + \frac{2}{T_2} Q_{\rm v} = {\rm i}\kappa_{\rm q} E_{\rm l} E_{\rm l}^*, \tag{3}$$

$$\frac{\partial n_{\rm a}}{\partial t} + \frac{n_{\rm a}}{T_1} = \frac{{\rm i}\omega_{\rm l}\kappa_{\rm l}}{4N\hbar} |E_{\rm l}|^2 (Q_{\rm v}^* - Q_{\rm v}), \tag{4}$$

$$\varepsilon_{\rm F} \propto n_{\rm a}, \tag{5}$$

with a delayed time $t = t' - z/v$, where v is the group velocity of femtosecond pulse propagation; $E_{\rm l}$ is the instantaneous field of exciting pulse deducted scattering from the medium; $n_{\rm a}$ and $Q_{\rm v}$ are the upper state population and the amplitude of collective vibration, which induces TPEF in biological tissues, respectively; $\varepsilon_{\rm F}$ is the energy of TPEF; $\kappa_{\rm s}$ and $\kappa_{\rm q}$ are coupling constants; T_1 and T_2 are the population relaxation time and dephasing time of two-photon excitation, respectively; N is the number density of molecule. Equation (2) may be solved for arbitrary initial conditions using Riemann's method. A special solution is the Bessel function. The initial condition of interest is that no vibration excitation exists at the beginning of the pulse. Thus, $Q(z) = 0$ at $t' \to -\infty$. By definition, $w(t') = \int_{-\infty}^{t'} |E_{\rm l}(t)|^2 {\rm d}t$, where $w(t')$ is the integral energy of exciting pulse at the time of t'. Equatipn (2) can be solved with arbitrary initial conditions. In the present case, the solution takes the form of

$$Q_{\rm v}(t) = -i\kappa_{\rm q} \int_{-\infty}^{t} \exp[-(t-t')/T_2] |E_{l_0}(t')|^2 \cdot J_0\{2[\kappa_{\rm l}\kappa_{\rm q}(w(t') - w(t'')L]^{1/2}\} {\rm d}t', \tag{6}$$

where $E_{l_0}(t)$ represents the initial value of exciting pulse fields, which will attenuate due to two-photon absorption; L is the effective length of the two-photon absorption; $J_0(x)$ is the zero-order Bessel function. The analytical solution of Eq. (2) is nonexistent. A reasonable approximation is made to neglect the depletion of the exciting pulse. Since $T_0 << T_2$, where T_0 is the width of the femtosecond pulse, the factor $\exp[-(t-t')/T_2]$ can be approximated by 1 in the integral of Eq. (6). Thus, in terms of the properties of the Bessel function, ${\rm d}J_1(x)/{\rm d}x = xJ_0(x)$, Eq. (6) gives

$$Q_{\rm v}(t') = \frac{Gw(t')^{1/2} \cdot J_1(Gw(t')^{1/2})}{2i\kappa_{\rm l}}, \tag{7}$$

where $J_1(x)$ is the first-order Bessel function; $G = 2\sqrt{\kappa_l \kappa_q L}$ characterizes the capability of two-photon absorption. For the very low effect of TPEF in biological tissue, $G\sqrt{w(t)} << 1$, the Bessel function takes the following form:

$$J_1(Gw(t)^{1/2}) \approx \frac{Gw(t)^{1/2}}{2} - \frac{G^3 w(t)^{3/2}}{16}. \tag{8}$$

Substituting Eq. (8) into Eq. (4) and integrating it yields

$$n_{\rm a} = \frac{\omega_l}{4N\hbar} \int_{-\infty}^{\infty} \exp(-t/T_1) |E_l(t)|^2 G^2 w(t) \Big[1 - \frac{G^2}{8} w(t)\Big] {\rm d}t. \tag{9}$$

Regarding the incident laser as a Gaussian pulse—namely,

$$|E_{l_0}(t)|^2 = I_0 \exp[-(t/T_0)^2], \tag{10}$$

where I_0 is the peak intensity of an exciting pulse. Since $T_0 << T_1$, the factor $\exp(-t/T_1)$ can be approximated by 1 in the integral of Eq. (9). Substituting Eq. (10) into Eq. (9) and combining it with Eq. (5), we get

$$\varepsilon_{\rm F} = A(\varepsilon^2 - B\varepsilon^3), \tag{11}$$

with

$$A = \frac{\omega_{\rm l} G^2}{4N\hbar} \int_{-\infty}^{+\infty} \exp[-(t/T_0)^2] \int_{-\infty}^{t/T_0} \exp[-(t'/T_0)^2] \cdot {\rm d}\Big(\frac{t'}{T_0}\Big) {\rm d}\Big(\frac{t}{T_0}\Big),$$

$$B = \frac{G^2}{8A} \int_{-\infty}^{+\infty} \exp[-(t/T_0)^2] \left\{ \int_{-\infty}^{t/T_0} \exp[-(t'/T_0)^2] {\rm d}\Big(\frac{t'}{T_0}\Big) \right\}^2 \cdot {\rm d}\Big(\frac{t}{T_0}\Big),$$

where $\varepsilon = I_0 T_0$ notes the energy of a single exciting pulse. Constant A or B depends on the properties of both the biological tissue and exciting pulse. Equation (11) shows that the energy of TPEF depends on the energy of the pulse instead of the intensity, which is just the characteristic of the transient process. In addition, the energy of TPEF is also dependent on the cube of the pulse energy.

To verify the result of Eq. (11), we chose a disease-free human thyroid as the specimen to be measured, which has frequently been investigated before. Specimens were obtained from the pathology laboratory of Fujian Provincial Hospital and immediately made into frozen (below –25 °C) slices.

Figure 2 shows a schematic of the experimental system. The specimens are excited at 830 and 850 nm. All spectra and images were collected using a Zeiss LSM 510 META laser scanning microscopy equipped with a mode-locked Ti:sapphire laser with a pulse width of approximately 110 fs at a 76-MHz repetition rate, tunable from 700 to 980 nm (Coherent Mira 900-F). The power of the exciting pulse illuminating the specimen was measured using a Coherent Model LM-10 HTD power detector. We detected all signals in the backward direction by the META detector, which consists of a high-quality, reflective grating and an optimized 32-channel photomultiplier tube (PMT) array detector, which covers a spectral width of approximately 340 nm ranging from 377 to 716 nm. The Lambda mode can obtain emission spectra from 425 to 716 nm in the region of interest.

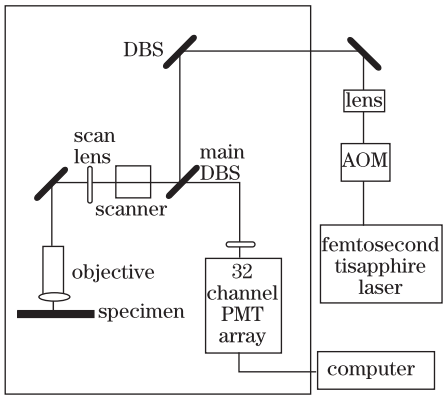

Fig. 2. Experimental setup for measuring the power of TPEF.

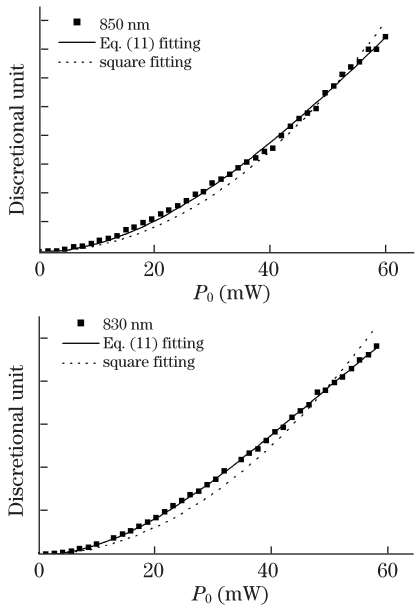

Fig. 3. Measured power of TPEF versus power of exciting pulse for human thyroid. P_0 indicates the power of exciting pulse illuminating on the specimen. The solid and dashed curves are plotted based on Eq. (11) and squarely law of pulse power fitting the data measured, respectively.

Considering that the power of TPEF is in proportion to the energy of the pulse (with the repetition rate as a proportionality constant), Eq. (11) can be tested by measuring both the power of TPEF and the exciting pulse. Figure 3 shows the dependence of the measured power of TPEF on the power of the exciting pulse. This measured data of TPEF is the sum of the wave ranging from 436 to 714 nm, which is mostly attributed to TPEF from the biological tissue. Figure 3 exhibits good agreement between the measured results and calculated curve based on Eq. (11). In contrast, the power of TPEF as a function of P_0^2 is also given in Fig. 3. The upper limit value of the illuminating power is subject to the dynamic range of the META detector.

In conclusion, we investigate the TPEF in biological tissue using a femtosecond pulse based on transient two-photon absorption. The dependence of TPEF on the power of the exciting pulse in the biological tissue may be derived by solving the set of coupled equations for transient two-photon absorption and confirmed by measuring the powers of both TPEF and the exciting pulse. Good agreement is obtained between the measured results and theory presented here. We believe that the result given here is useful to further study the non-linear effect in tissue optics.

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