Reduction of higher-order photobleaching in two-photon excitation microscopy

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A theoretical microscopic technique is proposed that may reduce multiphoton interaction in the excitation volume of a two-photon microscope. Since higher-order photobleaching is common in two-photon excitation microscopy, the study of thin samples is limited by increased photobleaching and photodamage. This limitation is elevated by using even coherent state light. The advantage of even coherent state light is that only excitation due to an even number of photons can survive. The very first nonzero even excitation (two-photon) can be isolated from the nearby one- and three-photon excitation. Hence the photobleaching due to one- and three-photon excitation can be eliminated and higher-order processes can be minimized owing to their small molecular cross section.

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Since the first demonstration of two-photon microscopy [1], the field of microscopy has undergone a dramatic improvement and caused a better understanding of life sciences at cellular and molecular level [2–4]. The exclusive occurrence of two-photon excitation at the focal volume of the microscope has the advantage of minimizing out-of-focus photodamage and photobleaching [5]. This is a decisive factor for imaging and also the limiting factor for long time monitoring of live cell and related cellular processes. Two-photon excitation microscopy is preferred for imaging applications because it has small and confined excitation volume compared to one-photon excitation microscopy [6]. The intensity squared dependence of two photon excitation microscopy is the secret behind its space localization [1]. But, high photon flux used in two-photon excitation microscopy lead to increased higher-order photon interactions as well in the focal volume. Research has shown that such a multiphoton interaction is significant and may lead to photobleaching and photodamage of the biological samples [7–9]. Possible mechanisms that can lead to higher-order dependence of photo bleaching are (1) coherent three- and higher-order-photon excitation of upper electronic states and (2) consecutive pumping of higher states via intermediate states. Although other physical processes do exists that may result in photodamage and photobleaching [11]. In this paper, we address the photobleaching and photodamage due to higher-order interaction processes in the focal volume of two-photon excitation microscopy.

Due to multiphoton excitation in the focal volume, deviation from intensity squared dependence is observed in many experiments [7,8]. The fluorescence intensity versus one- and two-photon excitation power is shown to increase with a slope of 1 and 2, respectively. But the two-photon photobleaching rate increases with a slope greater than 3, thus indicating the presence of higher-order photon interactions [7]. Experiments on Indo-1, NADH, and aminocoumarin produced similar results and suggest that this higher-order photobleaching is common in two-photon excitation microscopy [7]. Hence the use of two-photon excitation microscopy to study thin biological samples may be limited by increased photobleaching and photodamage. Previous experimental evidence suggests that photobleaching in the focal volume is more pronounced under two-photon excitation than with conventional microscopy [10,11]. Particularly, nicotinamide adenine dinuclestide (NADH) suggested that the photobleaching rate depended not on the square of the intensity, but also on a higher-order relationship [10,11]. Nonlinear photo switching is also reported for *A* and *B* forms of a GFP mutant [12].

At low intensity levels, thermally assisted one-photon excitation could become significant if the wavelength of two-photon absorption band overlaps the one-photon absorption band. This causes a severe departure from the square-law dependence. For example, a significant deviation from square-law for rhodamine B at intensity levels of approximately 10^{24}–10^{25} photons cm^{-2} s^{-1} for $\lambda < 730$ nm is reported in the literature [13].

For Ca^{++}-bound species, 2PE dominates at $\lambda < 830$ nm and 3PE at $\lambda > 900$ nm, while for Ca^{++}-free species, 2PE dominates up to 910 nm and 3PE only at $\lambda > 960$ nm. At intermediate wavelengths, a mixture of two- and three-photon excitation was observed [14]. Slopes in the logarithmic plot of fluorescence output versus intensity from 2.0 to 3.0 indicate that a mixture of 2PE and 3PE fluorescence was generated [14]. Also, the quadratic power dependence of 2PE microscopy does not hold under the saturation condition [15]. Further, it is often difficult to distinguish multiphoton excitation spectra especially two-photon from one- and threephoton excitation spectra because the same initial excited states can be reached via one/two/three photon excitation, without violating any selection rules [16].

The increase of signal to noise (SNR) ratio is possible if the input intensity increases without causing photobleaching and saturation. It should be noted that photobleaching and saturation occurs probably due to high-order photon interaction processes. So, it becomes essential to isolate 2PE from 1PE and 3PE. Other higher-order excitation can be neglected owing to its small molecular cross section and occurrence

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probability. Recently, Tryptophan and Serotonin were imaged with infrared illumination by three-photon excitation (3PE) [17]. The cross section of 3PE of these molecules are very small and hence excitation is difficult. Excitation with even coherent state in 2PE microscopy can improve the excitation probability without causing unwanted photodamage and photobleaching.

In this paper, we theoretically address conditions for possible isolation of two-photon excitation from one-photon and three-photon excitation. We propose to employ even coherent state light, for which only even number of photons have nonzero probability of being observed. Generation of even coherent state is reported in the literature [19–22]. Generally, the macroscopic superposition states (Schrödinger cat states) are generated by the interaction of two modes of the optical fields. Two modes of the field interact dispersively in a Kerr nonlinear medium, resulting in the entanglement of the resulting field state. Large amplitude of the cat states is reported in Jeong *et al.* [20,21]. It is possible to efficiently generate even coherent state by parametric down conversion [20–22]. Particularly, for generating the two-photon state by parametric down conversion process, a nonlinear crystal transforms the part of the incident photon energy to photons with half the energy. An important property of the down-converted light is the high degree of correlation, i.e., the detection of a photon at one point in space-time enhances the probability of the detection of its entangled photon at another space-time location [18].

It is expected that the light source for the two-photon excitation experiment requires lower photon flux compared to the flux of random source of light [22]. As an example, a typical nonlinear crystal can spontaneously down convert light with a typical efficiency of around 10^{-7}. Hence, about 10 W of pumping light is needed to generate 1 μW of down-converted power [23]. An efficient generation of even coherent state light may lead to better signal-to-noise ratio in two-photon microscopy.

For the sake of completeness and better understanding of the proposed microscopic technique, we briefly introduce nonclassical states of light and even coherent state light. The state of quantum mechanical systems such as coherent photon systems is characterized by the expectation of the moments of bosonic operators $\hat{a}$ and $\hat{a}^\dagger$, which are either in normally ordered form $\langle(\hat{a}^\dagger)^m(\hat{a})^n\rangle$, antinormally ordered form $\langle(\hat{a})^n(\hat{a}^\dagger)^m\rangle$, or symmetrical form $\langle\{(\hat{a}^\dagger)^m(\hat{a})^n\}\rangle$, where $\{\cdots\}$ represents Poisson bracket.

The general form of the s-parametrized characteristic function is given by

$$C(\xi,s) = \mathrm{tr}[\hat{\rho}e^{[\xi\hat{a}^\dagger - \xi^*\hat{a} + s(|\xi|^2/2)]}] \tag{1}$$

and the density operator $\hat{\rho}$ is the inverse Fourier transform of the characteristic function [24].

The expectation value of operator $\langle\{(\hat{a}^\dagger)^m(\hat{a})^n\}\rangle$ can be written as

$$\langle\{(\hat{a}^\dagger)^m(\hat{a})^n\}\rangle = \int W(\beta,0)\beta^{*m}\beta^n d^2\beta, \tag{2}$$

where the corresponding Wigner function is given by

$$W(\beta,s=0) = \frac{1}{\pi^2}\int C(\xi,s=0)e^{(\beta\xi^* - \beta^*\xi)}d^2\xi. \tag{3}$$

Consider the superposition of two coherent states $|\alpha\rangle$ and $|-\alpha\rangle$ with zero phase for generating even coherent states [25]

$$|\psi\rangle = \frac{1}{[2(1+e^{-2\alpha^2})]^{1/2}}(|\alpha\rangle + |-\alpha\rangle). \tag{4}$$

The Wigner function corresponding to the even coherent state is [25]

$$W(\beta) = W(\beta,s=0) = \frac{1}{\pi(1+e^{-2\alpha^2})}[e^{-2(x-\alpha)^2-2y^2} + e^{-2(x+\alpha)^2-2y^2} + e^{-2x^2-2y^2}\cos(4y\alpha)], \tag{5}$$

where $x=\mathrm{Re}(\beta)$ and $y=\mathrm{Im}(\beta)$. The last term arising from the quantum interference of $|\alpha\rangle$ and $|-\alpha\rangle$ is responsible for the nonclassical behavior of even coherent state light.

The quantum interference between the states $|\alpha\rangle$ and $|-\alpha\rangle$ generates an oscillatory behavior in the photon number distribution. The distribution function is given by [25]

$$P_n = \frac{2e^{-\alpha^2}}{1+e^{-2\alpha^2}}\frac{\alpha^{2n}}{n!} \quad \text{if } n=2m, \quad m=1,2,\ldots,$$

$$P_n = 0 \quad \text{if } n=2m+1, \quad m=0,1,\ldots. \tag{6}$$

This photon number distribution represents even coherent state because only even number of photons have nonzero probability of being observed. This is similar to bunching $2n$ photons together. It can be shown that even coherent state light has super-Poissonian photon statistics for any value of intensity α^2. The corresponding Mandel Q parameter is positive, i.e., $Q=4\alpha^2e^{-2\alpha^2}/(1-e^{-4\alpha^2})>0$.

In general, fluorophores in the focus volume can undergo more than single mode excitation, i.e., they may undergo one-photon, two-photon, or multiphoton excitation (depending upon the molecular cross section of the sample and excitation probability). The total fluorescent signal from the fluorophore molecules in the elemental volume element ΔV undergoing all possible modes of excitation processes may be expanded on the basis of the excitation mode, i.e.,

$$\begin{aligned} I_{fT}(\Delta V) &= I_{f(1)}(\Delta V) + I_{f(2)}(\Delta V) + I_{f(3)}(\Delta V) + \cdots \\ &= \delta_1 P_1 N_{\Delta V} + \delta_2 P_2 N_{\Delta V} + \delta_3 P_3 N_{\Delta V} + \cdots \\ &= \delta_i P_i N_{\Delta V} + \sum_{j\neq i}\delta_j P_j N_{\Delta V}, \end{aligned} \tag{7}$$

where $I_{f(1)}(\Delta V)$ represents ith order process, P_i is probability for i-photon excitation, $N_{\Delta V}$ is the number of fluorescent molecules in the focal volume ΔV, and δ_i is the molecular cross section corresponding to ith order excitation. Theoretically, all the modes of excitation are possible, but the relative probability of higher-order processes drops drastically owing to its low molecular cross section.

A coherent state of light with complex amplitude α has a photon number distribution given by Poisson distribution. This simply states that the probability $P(n)$ to find n photons

in a single state $|\alpha\rangle$ is given by Poisson distribution, i.e.,

$$P(n) = |\langle n|\alpha\rangle|^2 = \frac{e^{-\langle n\rangle}\langle n\rangle^n}{n!}, \tag{8}$$

where $\langle n\rangle = |\alpha|^2$ is the average number of photons equal to the classical intensity of the coherent light.

On the other hand, photons exhibiting quantum interference have an oscillating behavior in the photon number distribution. This means that there exists nonzero probability of observing bunch of even photons and zero probability of observing odd photons or vice versa. Such a behavior is classically impossible. The probability of finding two photons $(n=2)$ in such a mixed superposition of coherent states $|\alpha\rangle$ and $|-\alpha\rangle$ exhibiting even coherent states is given by [Eq. (6)]

$$P_2 = \frac{2e^{-\alpha^2}}{1+e^{-2\alpha^2}}\frac{\alpha^4}{2!} = \alpha^4 \,\mathrm{sech}(\alpha^2). \tag{9}$$

It should be noted that this does not deny the possibility of the existence of higher even order bunching of photons (i.e., $P_n \neq 0$ for $n=2m$, $m>1$).[1]

Series expansion of Eq. (10) produces $P_2 = \alpha^4\{1-\frac{1}{2}\alpha^4+\frac{5}{24}\alpha^8+\cdots\}$. For small excitation intensity $I=\langle n\rangle=\alpha^2$, practically true for two-photon microscopy [15,2], $\mathrm{sech}(\alpha^2)\approx 1$, and thus

$$P_2 \propto \alpha^4 = C_I I^2, \tag{10}$$

where C_I is a proportionality constant. It should be noted that excitation by even coherent state light preserves the intensity squared dependence property of two-photon microscopy.

We propose to excite fluorescent molecules using even coherent state light. It is assumed that the experimental conditions are such that the during excitation of the fluorescent molecules by the coherent two-photon superposition states; the pumping of the upper molecular levels via intermediate virtual levels by secondary two-photon excitation is ruled out. This means that as long as the molecule remains in the singlet systems, the molecule is protected from photobleaching.

Now the explicit expression for the total fluorescent signal for a pulsed laser beam with pulse width τ_p, repetition rate f_p, average power P_{av} and the effective power $P(t)(=P_{\mathrm{av}}/\tau_p f_p$ for $0<t<\tau_p)$ [1,2,15] is given by

$$I_{f(2)}(\Delta V,t) = \delta_2 \frac{P_{\mathrm{av}}^2}{\tau_p f_p^2}\left[\frac{\pi(NA)^2}{hc\lambda}\right]^2 N_{\Delta V}, \tag{11}$$

where NA is the numerical aperture of the objective lens.

The probability of finding four photons in such a mixed superposition of coherent states is

$$P_4 = \frac{2e^{-\alpha^2}}{1+e^{-2\alpha^2}}\frac{\alpha^8}{4!} = \frac{1}{12}\alpha^8 \,\mathrm{sech}(\alpha^2). \tag{12}$$

In the small intensity approximation, $[\mathrm{sech}(\alpha^2)=1]$; thus

$$P_4 \propto \alpha^8 = I^4. \tag{13}$$

Generally for even coherent state the intensity dependence may be shown to be $P_n \propto I^n$.

The fluorescence intensity $I_{f(4)}(t)$ for possible four-photon excitation is proportional to the molecular cross section δ_4, $I(t)^4$, and $N_{\Delta V}$, i.e.,

$$I_{f(4)}(t) \propto \delta_4 I(t)^4 N_{\Delta V} \propto \delta_4 P(t)^4 \left[\frac{\pi(NA)^2}{hc\lambda}\right]^4 N_{\Delta V}. \tag{14}$$

Assuming pulsed laser, the four-photon absorption by a single fluorophore molecule during a single pulse can be approximately given by

$$I_{f(4)} \propto \delta_4 \frac{P_{\mathrm{av}}^4}{\tau_p^3 f_p^4}\left[\frac{\pi(NA)^2}{hc\lambda}\right]^4 N_{\Delta V}. \tag{15}$$

It can be shown that the molecular cross section for nth order process, i.e., n-photon interaction with a single fluorophore, is given by [16] $\delta_n \cong \delta_1^n \Delta t^{n-1}$, where the time interval Δt is the time scale of molecular energy fluctuations at photon energy scales, as determined by the Heisenberg uncertainty principle: $\Delta t \cong 10^{-17}$ sec. Approximate value of δ_1 is 10^{-17} cm^2. So the molecular cross section for four-photon excitation is of the order of 10^{-119}. It should be noted that molecular cross section depends upon the sample [16].

The probability of two-photon and higher even order processes follows: $P_2 \gg P_4 \gg \cdots \gg P_{2m}$ in the small intensity approximation. Hence the fluorescence signal follows:

$$I_{f(2)} \gg I_{f(4)} \gg \cdots \gg I_{f(2m)}. \tag{16}$$

Since one-photon and three-photon belongs to odd coherent states, the corresponding excitation probability becomes zero, i.e., $P_1=P_3=\cdots=P_{2m+1}=\cdots=0$, resulting in

$$I_{f(1)} = I_{f(3)} = \cdots = I_{f(2m+1)} = 0. \tag{17}$$

Hence the total fluorescent signal in the elemental volume using even coherent state light is essentially due to two-photon excitation and can be approximated as

$$I_{fT}(\Delta V) \approx \delta_2 P_2 N_{\Delta V} \approx \delta_2 \frac{P_{\mathrm{av}}^2}{\tau_p f_p^2}\left[\frac{\pi(NA)^2}{hc\lambda}\right]^2 N_{\Delta V}. \tag{18}$$

With even coherent state light, the dependence of two-photon excitation on I^2 is preserved in the low intensity limit and the effect of two-photon excitation is potentially isolated from the neighboring excitation modes, i.e., one-photon and three-photon excitation. It should be noted that though other even order excitation processes $(n=4,6,\ldots,2m)$ are possible in the focal volume, but have very low occurrence probabilities.

In this paper, we proposed an approach that may reduce photobleaching due to one and multiphoton excitation in two-photon excitation microscopy. To achieve this, we use even coherent state light arising due to quantum interference between two coherent states. Such a light has the advantage

[1]This is beneficial for two-photon excitation because of the $2n$-photon nature of even coherent state. So, if the detection window is configured to detect photons arising due to two-photon excitation, then the neighboring odd states (1 and 3 photon states) and other higher even order processes can be blocked. Moreover, the probability of higher-order excitation is negligible compared to two-photon excitation.

that the probability of observing odd coherent state is zero and there exists nonzero probability for observing even photons. This is of great impact when using visible wavelength for producing nonlinear effects.

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