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Multiple stimulated emission fluorescence photoacoustic sensing and spectroscopy

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Multiple stimulated emission fluorescence photoacoustic (MSEF-PA) phenomenon is demonstrated in this letter. Under simultaneous illumination of pumping light and stimulated emission light, the fluorescence emission process is speeded up by the stimulated emission effect. This leads to nonlinear enhancement of photoacoustic signal while the quantity of absorbed photons is more than that of fluorescent molecules illuminated by pumping light. The electronic states' specificity of fluorescent molecular can also be labelled by the MSEF-PA signals, which can potentially be used to obtain fluorescence excitation spectrum in deep scattering tissue with nonlinearly enhanced photoacoustic detection. In this preliminary study, the fluorescence excitation spectrum is reconstructed by MSEF-PA signals through sweeping the wavelength of exciting light, which confirms the theoretical derivation well. *Published by AIP Publishing.* [<http://dx.doi.org/10.1063/1.4955096>]

Fluorescence technology has been widely used in biological sciences. Fluorescence spectroscopy, time-resolved fluorescence, and fluorescence imaging can be, for instance, used to diagnose cancer and monitor the progress of cancer treatment.^{1–6} Because of the strong optical scattering in tissue, the photons propagate diffusely inside the tissue. As a result, it is hard to distinguish the spatial origins of fluorescence signals and achieve good spatial resolution deep in scattering medium. To overcome this obstacle, the photoacoustic (PA) technology,^{7–12} which combines optical excitation with ultrasonic detection, has been developed, circumventing the optical diffusion limit. Razansky *et al.*¹³ described a multispectral optoacoustic tomography (MSOT) technique capable of high resolution visualization of fluorescent proteins deep within highly scattering living organisms. Wang and Wang¹⁴ have reported Förster Resonance Energy Transfer (FRET) photoacoustic microscopy based on non-radiative decay that produces heat and subsequent acoustic waves, which however needs the donor and acceptor pair with a half maximum distance in the 1–10 nm range. Morgounova *et al.*¹⁵ has focused on a contrast mechanism for photoacoustic imaging based on the difference in excited-state lifetime between monomer and dimer forms of a chromophore. Märk *et al.*¹⁶ introduced a pump-probe technique for the detection of fluorophores in tomographic PA images based on inducing stimulated emission in fluorescent molecules, which in turn modulates the amount of thermalized energy, and hence the PA signal amplitude. As the stimulated emission in fluorescent molecules is only a one time process, the PA signal induced by transforming thermalized energy from this process is relatively weak.

In this letter, we present a multiple stimulated emission fluorescence (MSEF) photoacoustic (MSEF-PA) phenomenon. Märk's single stimulated emission process and MSEF effect presented in this paper are different cases, where the process of stimulated emission is exploited to enhance the PA signal. The increased PA signal in MSEF-PA compared to SEF-PA is determined by the multiple repetition process and key laser parameters (pulse energy, pulse width, and wavelength). Theoretical prediction of the enhancement cannot be described in formula but needs numerical calculations. The SEF is part of the reversible saturate optical fluorescence transition concept¹⁷ and was pioneered by Hell and Wichmann.¹⁸ It has been used to overcome the optical diffraction limit in microscopy.¹⁹ However, the generation of photoacoustic pressure wave induced by the laser pulses in the MSEF progress has not been addressed yet. In this study, the MSEF-PA starts from the premise that the quantity of absorbed photons is more than that of fluorescent molecules illuminated by pumping light, the photoacoustic is not only induced by the linear absorption of lights, but also enhanced by the phonons produced in the repeated stimulated emission process. We derive the relationship between the increment of MSEF non-radiative decay energy and the parameters of both laser and fluorescent molecule based on kinetic equation.²⁰ Then, we will reconstruct the fluorescence spectrum of Ce⁺:YAG by the nonlinear increment of MSEF photoacoustic signals, which agrees with our derivation well. However, the further application of MSEF-PA for bio-imaging in deep tissue will be studied elsewhere.

The photoacoustic signal induced by the non-radiative decay in a tissue sample can be described by the use of Jablonski diagram. A typical Jablonski diagram is shown in Figure 1(a).²⁰ The singlet ground, first excited, and second excited electronic states are depicted by S_0 , S_1 , and S_2 , respectively. At each of these electronic energy levels, the fluorophores can populate in a number of vibrational energy levels,

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FIG. 1. (a) The spontaneous fluorescence energy-level diagram for a molecular system, (b) The principle of MSEF PA effect. VR: Vibrational Relaxation; ic: internal conversion.

depicted by 0, 1, 2, etc. The lifetimes of those states are also annotated in Figure 1(a). According to the Franck-Condon principle, absorption occurs so fast that there is no time for molecular motion during the absorption process. It occurs in less than 10^{-15} s. A fluorophore is usually excited to some higher vibrational level of either S_1 or S_2 , with a few relaxed and fast relaxation steps to the lowest vibrational level of S_1 . This process is called internal conversion and generally occurs within 10^{-12} s or less. The intersystem crossing happens at low rate and it is usually neglected.

Assuming that the vibrational ground state density is N_0 , and the first and second excited state densities are N_1 and N_2 , respectively, these densities can be calculated based on the kinetic equation²⁰

$$dN_0/dt = -\sigma_{01}\Phi_p N_0 - \sigma_{02}\Phi_p N_0 + \sigma_{10}\Phi_e N_1 + k_{ic}N_1 + k_f N_1, \quad (1a)$$

$$dN_1/dt = \sigma_{01}\Phi_p N_0 - \sigma_{10}\Phi_e N_1 + k_{ic}N_2 - k_f N_1 - k_{ic}N_1, \quad (1b)$$

$$dN_2/dt = \sigma_{02}\Phi_p N_0 - k_{ic}N_2, \quad (1c)$$

$$N_g = N_0 + N_1 + N_2, \quad (1d)$$

where the quantities k_f, k_{ic} represent the transition rates for fluorescence emission and internal conversion, respectively. Their inverses denote the approximate value of transmit lifetime.²¹ σ_{01} and σ_{02} are the absorption cross sections from ground to first and second state, respectively. Φ_p and Φ_e are photon fluxes for absorption and stimulated emission, respectively. N_g is the total quantity of the molecular on the ground vibrational levels.

In the case of illumination with pumping laser only, the number of photons from the laser is also far more than the density of the molecules in the ground energy level. During a one nanosecond laser pulse, the absorption process and the internal conversion are much faster than fluorescence emission process. All of molecules are excited to vibrational levels and relax to first excited singlet state in short time, which is much shorter than the S_1 lifetime. Under high pumping laser intensity, the molecules relaxing from the first state will be pumped again and relax to the first excited singlet state in a short time. This pumping and relaxation will be repeated again and again. Equation (1b) can be approximately written as

$$dN_1/dt = k_{ic}N_2 - k_f N_1 = k_{ic}k_f N_1 - k_f N_1, \quad (2)$$

and the solution for Equation (2) is

$$N_1 = N_g e^{-k_f(1-k_{ic})t} \approx N_g e^{-(\tau_{ic}-1)t/\tau_{ic}\tau_f}. \quad (3)$$

Here, τ_f and τ_{ic} represent the transition time for fluorescence emission and internal conversion, respectively. Due to the pumping, photonic energy is not enough to third, fourth, or higher electronic state, the quantity of molecules relaxing from higher vibrational levels, that is mainly from second electronic state, to first energy level consists of two parts: all the molecules (N_g) are excited for the first time and part of molecules ($\int_0^{\tau_p} (N_1 k_f) dt$) is excited for the second time. So, we can have the non-radiative decay energy per pumping laser pulse from the Eq. (3) and the energy difference of first and second electronic states as

$$\begin{aligned} E_f &= \hbar(\omega_p - \omega_f) \left[N_g + \int_0^{\tau_p} (N_1 k_f) dt \right] \\ &= \hbar(\omega_p - \omega_f) N_g [1 + \tau_{ic} e^{\tau_p/(\tau_{ic}\tau_f)}]. \end{aligned} \quad (4)$$

Here, ω_p and ω_f are the puming and emission frequency, respectively, and τ_p represents the pulse width of pumping light. Together with pumping light, we simultaneously utilize another pulsed laser with a wavelength near to the fluorescence maximum of the fluorophore. As the lifetime of absorption process is far less than the other processes, all of molecules will be excited to vibrational levels in a short time under high pumping power intensity. The temporal evolution of occupation levels in Equation (1) can be approximately written as

$$dN_1/dt = k_{ic}N_2 - \sigma_{10}\Phi_e N_1, \quad (5a)$$

$$dN_2/dt = -k_{ic}N_2 + \sigma_{10}\Phi_e N_1, \quad (5b)$$

$$N_1(0) \approx 0, \quad N_2(0) \approx N_g. \quad (5c)$$

As the lifetimes of absorption process and internal conversion are far smaller than the laser pulse duration in a few nanoseconds, the Φ_e changes with the time slowly compared to stimulated emission process. Its solution is thus approximately given as

$$\begin{aligned} N_1 &= N_g [1 - e^{-(\Phi_e \sigma_{10} + k_{ic})t}] k_{ic} / (\Phi_e \sigma_{10} + k_{ic}) \\ &\approx N_g (1 - e^{-t/\tau_{ic}}). \end{aligned} \quad (6)$$

According to the definition of fluorescence lifetime¹⁹ and Eq. (6), the transition lifetime for the stimulated fluorescence emission process of τ_{sf} is similar with τ_{ic} , which is less than 10^{-11} s. It is shown in Fig. 1(b) that the fluorescence emission process will be speeded up and the lifetime will decrease from 10^{-8} – 10^{-10} s to less than 10^{-11} s, which is with the same order of magnitude as the internal conversion lifetime. Under the condition of high power intensity pumping light, the fluorophores are excited to high vibrational level and relaxed to the ground vibrational level with fluorescence emission, after that they will be pumped to high vibrational level again. This process will be repeated several times, and the energy of non-radiative decay will increase

during this process. The released energy from internal conversions is converted to the heating and thermoelastic vibration thereafter. Therefore, the stimulated emission fluorescence process will induce increased photoacoustic signals under the simultaneous illumination of pumping light (in high power intensity) and stimulated emission inducing light, both with nanosecond pulse width. The variation of molecular densities in Eq. (1) can be considered as a transient process compared to the repeated processes and can be neglected. The stimulated emission photons interacting with molecules contain two parts: the input stimulated emission inducing light and the photons induced by molecules transmitting from the first energy level to ground energy levels. Assuming N_f is the quantity of stimulated emission inducing light photons and $g(\nu)$ represents the molecular transition probability from first excited energy level to different ground state energy levels stimulated by the stimulated emission inducing light, and then the quantity of photons induced by this process could be expressed as $N_f g(\nu)$. Then, we can simplify and derive an approximate expression for the heating energy of MSEF-PA generation as

$$E_{sf} = \hbar(\omega_p - \omega_f)nN_f[1 + g(\nu)], \quad (7)$$

where n represents the number of repetition for the process of the fluorophores excited to high vibrational level and relaxed to the ground vibrational level, which is equal to $\tau_p/(\tau_{sf} + \tau_{ic})$.

We distinguish the MSEF induced PA signal from the linear absorption induced PA signal by subtracting photoacoustic signal induced by applying the two lasers at once from the summation of the photoacoustic signals linearly induced by stimulated emission inducing laser and pumping laser illuminations separately. Although the signal amplification factor is moderate in absolute value, the linear absorption induced background will be significantly suppressed after subtraction (ideally no background interference), which will be quite useful for visualizing fluorophores from the highly absorptive background. Then, we can obtain the increment of heating energy induced by the stimulated fluorescence process by subtracting Eq. (4) from Eq. (7)

$$\Delta E = \hbar(\omega_p - \omega_f)\{nN_f[1 + g(\nu)] - N_g[1 + \tau_{ic}e^{\tau_p/(\tau_{ic}\tau_f)}]\}, \quad (8)$$

hence the molecular transition probability $g(\nu)$ can be expressed in terms of ΔE from Eq. (8)

$$g(\nu) = \frac{\Delta E}{\hbar(\omega_p - \omega_f)nN_f} + \frac{N_g[1 + \tau_{ic}e^{\tau_p/(\tau_{ic}\tau_f)}] - nN_f}{nN_f} \\ = \frac{K\Delta p}{\hbar(\omega_p - \omega_f)nN_f} + \frac{N_g[1 + \tau_{ic}e^{\tau_p/(\tau_{ic}\tau_f)}] - nN_f}{nN_f}. \quad (9)$$

Here Δp is the increment amplitude of photoacoustic signals, K is the photoacoustic constant. Eq. (9) can be used to calculate the transition probability $g(\nu)$ of fluorophores, which plays a key role in the fluorescence spectroscopy, and it is proportional to the increment amplitude of MSEF photoacoustic signals. Based on it, if we sweep the exciting light

wavelength, we can tune the transition probability of molecules on different ground vibrational levels, which can be potentially used to reconstruct the fluorescence spectroscopy in deep scattering tissues with photoacoustic detection.

The experimental setup is shown in Fig. 2. A wavelength-tunable optical parametric oscillator (OPO) laser (Opolette-355I, Opolettek, Inc., USA) is used to provide the collimated pumping and stimulated emission inducing light sources. The pumping light has 3.5 mJ pulse energy, 7 ns of pulse width, and 355 nm wavelength and is generated by the third-order harmonic generation (THG) effect of 1064 nm laser. The wavelength-tunable light output with ~ 3 mJ pulse energy, 7 ns pulse width, and 480–670 nm wavelength is used as simulated emission inducing light. The pumping light is attenuated by a neutral density filter (NDC-50C-2M, Thorlabs). Then, it passes through a 355 nm band-pass filter (FL355-10, Thorlabs). Both laser beams are combined and collimated by a beam splitter. A focused ultrasound transducer (V323-SU, Olympus) with a 3.5 MHz central frequency is used to detect the PA signal, followed by a 54 dB gain pre-amplifier (5662, Olympus). The PA signal is recorded by a digital oscilloscope (WaveRunner 640Zi, LeCroy) with 5 GHz sampling rate, and sent to a personal computer for post-processing. The powder of Ce+:YAG (YAG-02, Intermix, USA) with the density of 4.8 g/cm³ and the central fluorescence emission wavelength of 552 nm is filled in a cuvette (751–5 mm, Huafeng Gaoke Co., Ltd.) as sample, which is immersed in water for optimum light transparency and acoustic coupling.

The optical mounts are adjusted to obtain optimal overlay on the Ce+:YAG sample. First, we record the photoacoustic signal induced by the pumping and stimulated emission inducing light alone, respectively. Then, we obtain the MSEF photoacoustic signal when two beams are coupled and illuminated simultaneously on the confocal spot of the sample. The time-domain signals induced by the three setups of laser illuminations are shown in Fig. 3. The wavelengths of pumping and stimulated emission inducing light are 355 nm and 515 nm, respectively. Their average powers are 13.4 mW and 5.7 mW, respectively. It clearly shows that the dual laser induced photoacoustic signal is stronger than the summation of the photoacoustic signals induced by the stimulated emission inducing and pumping laser illuminations separately, which indicates

FIG. 2. MSEF photoacoustic experiment setup. THG: third-order harmonic generation; OPO: optical parametric oscillator.

FIG. 3. The photoacoustic signal's time-domain waveform induced by stimulated emission inducing, pumping laser separately (red and blue lines), their sum (green dotted line), and dual lasers illumination simultaneously (black line).

the non-linear increase in the photoacoustic signal caused by stimulated fluorescence emission process.

The quantity of photons per laser pulse of pumping light in the Ce+:YAG sample is about 6.25×10^{15} , which is obtained from 3.5 mJ of pulse energy divided by 5.6×10^{-19} J of 355 nm photon energy. As the Ce+:YAG density is 4.8 g/cm³, its formula weight is about 593.7 g/mol and Ce+ doped solubility is about 1%, the number of moles per cubic millimetre is 8.08×10^{-8} , then the quantity of fluorescent molecules per cubic millimetre is about 4.87×10^{16} . When the beam is focused into 0.5 mm of diameter (defined by FWHM) and the penetration depth is less than 0.125 mm, the total quantity of fluorescent molecules in the volume illuminated by the pumping and stimulated emission inducing lasers is around 1.19×10^{15} , which is less than the photons density per pulse (6.25×10^{15}). The increment of photoacoustic signal follows Eq. (8). In order to confirm the validity of Eq. (8), first we fixed the average power of pumping light and swept the average power of the 515 nm stimulated emission inducing laser; the dual lasers induced photoacoustic signal is subtracted by the sum of photoacoustic signals induced by pumping and stimulated emission inducing lasers separately. Then, we get the increment of photoacoustic signals induced by the MSEF proportional to the power of stimulated emission inducing light. With the same method, we get the relationship between the increment of photoacoustic signals induced by the MSEF and the power of pumping light. The results are shown in Fig. 4, which shows that the increment of photoacoustic signal is linearly proportional to the power of stimulated emission

FIG. 4. The relationship between MSEF photoacoustic normalized to the sum of the two single photon induced PA signals and the average power of lights: (a) stimulated emission inducing light; (b) pumping light.

inducing light, namely, $\Delta p \propto \Delta E \propto N_f$. In Fig. 4(b), it verifies the MSEF-PA generation that when the power of pumping light is low, namely, the quantity of pumping photons is less than that of molecules illuminated by the pumping light, the repeated excitation process cannot occur. So, that the increment of photoacoustic signal is nearly equal to zero. When the quantity of pumping photons have the same order of magnitude as that of molecules illuminated by pumping light, the increment of photoacoustic signal will increase with the power of pumping light, which enhance the repeat excited process. Finally, the increment of photoacoustic signal will reach a maximum value when above the threshold power of pumping light, because it is limited by the total quantity of fluorescent molecules.

In Eq. (9), the transition probability of fluorophores molecular $g(\nu)$ is proportional to the increment of photoacoustic signal induced by the MSEF phenomenon. In the experiment, the power of pumping laser is fixed above the threshold power and the wavelength of stimulated emission inducing light is swept in the range from 480 nm to 670 nm. According to Eq. (9), the increment of photoacoustic signal is calculated and shown in Fig. 5 (black dot).

Based on the laser spectroscopy theory, the $g(\nu)$ can be approximately formulated as²¹

$$g(\nu) = \frac{1}{2\pi} \times \frac{\Delta\nu}{(\nu - \nu_0)^2 + (\Delta\nu/2)^2}. \quad (10)$$

Here, ν , ν_0 are the transition frequency and centre transition frequency separately, $\Delta\nu$ is the transition linewidth (FWHM). We obtain the spectrum of YAG-02 utilizing the spectroscopy (CCS200, Thorlabs, Inc., USA) shown in Fig. 5 (black line). The central wavelength of YAG-02 sample is about 548.8 nm and its FWHM is about 89.9 nm, namely, the centre transition frequency and transition linewidth are 5.47×10^{14} Hz and 8.7×10^{13} Hz separately. Hence, the theoretical molecular transition probability can be calculated based on Eq. (10) and plotted in Fig. 5 (blue line). If using molecular transition probability as indicator for fluorescence spectrum, comparing the measured MSEF molecular transition probability with theoretical molecular transition probability in Fig. 5, the measured MSEF photoacoustic spectrum achieved a very good agreement with the theoretical fluorescence spectrum.

To develop the MSEF induced PA effect towards real biomedical application, we need to limit the laser fluence below the ANSI standard (<20 mJ/cm² for visible light). To

FIG. 5. Spectrum for Ce+:YAG utilizing the MSEF-PA, theoretical calculation and spectroscopy.

observe the MSEF induced PA effect at low laser fluence, the fluorescence molecular density should be kept low, so that the quantity of pumping photons could be more than that of molecules illuminated by pumping light. In this case, the PA signal enhancement could also be expected to be observed. However, the challenge is due to the low concentration of the samples and laser fluence, and the absolute amplitude of both linear absorption PA signal and MSEF induced PA will be weaker, which requires further system update for the measurement and will be explored in the future work. In addition, we believe that using pumping and probing light source at near-infrared wavelength will further push the MSEF induced PA effect towards real biomedical applications using suitable fluorophore for deep tissue sensing and imaging. And the feasibility of doing so is highly expected based on the previous work of near-infrared fluorophores used in biomedical sensing and imaging. Last, we could combine both fluorescence and PA intensity increasing to get more information of localization of PA signal and specificity of fluorescence spectroscopy, working as a dual-modal imaging method. We will study these interesting topics in our future work.

In conclusion, under simultaneous illumination of pumping light and stimulated emission inducing light, the MSEF photoacoustic phenomenon is theoretically modelled and experimentally observed. Moreover, the MSEF photoacoustic signal reflects the properties of the molecule's "fluorescence," which can be used for fluorescence spectrum. It paves the way to use the MSEF-PA technique for imaging the distribution of fluorophores in deep scattering medium. Last, we predict that if the molecules meet the requirement of the electronic energy structure and laser

parameters (like pulse energy, pulse width, and wavelength), this MSEF effect may appear in non-radiative material which needs some more study in future.

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