Squeezed Light Induced Two-photon Absorption Fluorescence of Fluorescein Biomarkers

1,3,*Tian Li, 2,3 Fu Li, 3,4 Charles Altuzarra, 3 Anton Glassen, 1,2,3 Girish S. Agarwal

1 Department of Biological and Agricultural Engineering, Texas A&M University, College Station, TX 77843

2 Department of Physics and Astronomy, Texas A&M University, College Station, TX 77843

3 Institute for Quantum Science and Engineering, Texas A&M University, College Station, TX 77843

4 School of Physics and Astronomy, University of Glasgow, Glasgow G12 8QQ, U.K.

*tian.li@tamu.edu
Two-photon absorption (TPA) microscopy is the most widely used technique for optically imaging tissues at depths down to several cell layers below the surface [1]. The TPA effect has several advantages such as reduced out-of-focus photobleaching, less autofluorescence, deeper tissue penetration and intrinsically high 3-dimensional resolution [2–5]. Unfortunately, classical TPA is an extremely inefficient process and scattering in biological tissues is very prominent. Therefore, TPA sensing and imaging generally requires the use of high photon-flux-density optical sources, most frequently femtosecond pulsed lasers, to insure that two photons have a significantly high probability of arriving simultaneously and effectively be absorbed [6, 7]. However, since the excitation power in each pulse is typically $10^5$ times more than its average power, it is very likely that the sample will endure significant photodamage such as phototoxicity or photobleaching [8, 9].

In parallel, photon pairs generated by using spontaneous parametric down-conversion (SPDC) have been used to demonstrate that the quantum temporal correlation characteristics are fitting for TPA [10–13]. However, works produced in such entangled TPA (ETPA) suffer from being limited to a low photon flux of $\sim 10^7$ photons/s, equivalent in the near-infrared wavelengths to $\sim 10$ pW, which is unviable for bioimaging and biosensing. This is mostly due to the low conversion efficiencies of nonlinear crystals used to generate the entangled photon pairs. It has also been shown that ETPA is unique in that it follows a linear behavior [10–16], unlike the quadratic behavior specific to classical TPA.

In this work, the light source is strongly quantum in nature and contains significantly higher entangled photon pair flux as compared to SPDC, of the order of $10^{13}$ to $10^{16}$ photons/s. This source is generated with four-wave mixing (FWM) in an atomic $^{85}\text{Rb}$
FIG. 1. Experimental setup and squeezing spectrum. (a) Squeezing spectrum of the twin beams measured by a radio frequency spectrum analyzer with a resolution bandwidth RBW of 300 kHz and a video bandwidth VBW of 100 Hz. The noise power of the intensity-difference of the twin beams is represented by the red line. The blue line is the standard quantum limit. -6.5 dB of squeezing is achieved. (b) Level structure of the D1 transition of $^{85}$Rb and the optical frequencies arranged in the double-Λ configuration. Here $\nu_p$ and $\nu_c$ are the twin beams, and $\nu_1$ is the pump. They satisfy the energy conservation condition, $\nu_p + \nu_c = 2\nu_1$. The width of the excited state in the level diagram represents the Doppler broadened line, $\Delta$ is the one-photon detuning, $\delta$ is the two-photon detuning, and $\nu_{HF}$ is the hyperfine splitting in the electronic ground state of $^{85}$Rb. (c) The molecular structure of DCM and fluorescein. (d) The imaging setup in which a ‘seeded’ Rb cell produces ‘twin beams’ via the FWM process. The strong quantum correlated twin beams are combined with a 10× objective onto the sample. Fluorescence is collected laterally with another 10× objective onto a photomultiplier tube (PMT). Two short-pass filters are mounted in front of the PMT to exclude the pump wavelength from being detected. The whole setup is enclosed in a light-proof box.
vapor (see Fig. 1(b) for the atomic level structure), which has a great capability of generating strong quantum-correlated beams of light (i.e., ‘twin beams’), also referred to as squeezed light [17]. Vapors of hot \(^{85}\text{Rb}\) atoms provide a medium in which FWM takes place. The medium has significant third order electric susceptibility \(\chi^{(3)}\), and when appropriately chosen laser light ‘seeds’ the medium, and squeezed light is produced (see Fig. 1(a) for a typical squeezing spectrum and experimental details can be found in Methods). Practically, FWM has proven to be an excellent platform for quantum sensing applications because of its great potential for generating squeezed light and entanglement, and for its experimental simplicity and integrability [18–22]. As can be seen from Fig. 1(a), the entangled photon pair flux of the source exhibits a strong intensity-difference squeezing of -6.5 dB, which is indicative of strong quantum correlations between the twin beams. Due to the much higher production of photon pairs in the squeezed light source and differently from the entirety of ETPA experiments governed by a low photon pair flux, we further report that the relationship between input power and fluorescence intensity with squeezed light follows a nonlinear behavior.

Fluorescein is one of the most frequently used biomarkers for bioimaging and biosensing [23]. Its small size is very convenient for \textit{in vivo} imaging, although its relatively small TPA cross-section generates low amounts of fluorescence [6, 7]. As shown in Fig. 1(d), the imaging system used to demonstrate squeezed light TPA (SL-TPA) of fluorescein is comprised of two \(10\times\) objectives for excitation and lateral collection, and is isolated from background disturbance with an enclosure. The operating wavelength incident on the fluorophores is \(\lambda = 795\) nm, which is effectively filtered out from the photo-multiplier tube (PMT) detection with optical low-pass filters. In addition, all measurements conducted with squeezed light are compared with measurements conducted with a continuous-wave (CW) laser of the same intensity, which in this work the classical coherent light source.

The classical TPA measurements of fluorescein (see Methods for its preparation) as shown in Fig. 2(a) by the red diamonds indicate that the power law follows a quadratic relationship. This confirms the theory for classical TPA fluorescence, where the fluorescence signal is proportional to the square of excitation light intensity. The measurements for SL-TPA are conducted with \(8\) mW of squeezed light (combined power of the twin beams) and its fluorescence is compared with that generated from \(8\) mW of coherent light. It can be observed from the figure that the fluorescence signal from \(8\) mW of coherent light excitation
FIG. 2. (a) Fluorescence signal versus coherent light excitation power, and enhancement of a factor of ~50 with squeezed light at 8 mW. The fluorescence signal from 4 mW of squeezed light excitation is also shown. Inset: raw fluorescence signals from PMT for coherent light (red) and squeezed light (green) excitations with 8 mW optical power. Shaded area for each curve represents one standard deviation. (b) Fluorescence signal induced by 8 mW of squeezed light with three different two-photon detunings, shown in the atomic level structure in Fig. [1]b) as δ. Red, green and blue bars are for δ = -10 MHz, -5 MHz and 0 MHz, respectively. This subfigure demonstrates degraded enhancements as a function of relative arrival times of the entangled photon pairs.

is characterized with a standard deviation for which the lower end is below zero. This is due to the relatively small classical TPA cross-section and due to the effect of background noise (e.g., electronic dark counts and spurious counts from stray ambient light) on the low fluorescence signal created from 8 mW of coherent light excitation. The experimental mean fluorescence signal is therefore affected as shown from its slightly displaced value as compared to the quadratic fit (dash-dotted red line), which represents the benchmark of the true value of measured fluorescence. However, when fluorescein is excited with 8 mW of squeezed light, the fluorescence signal (represented by the green star) is enhanced by a factor of ~50 as compared to the true value of TPA fluorescence with 8 mW of coherent excitation. The fluorescence signal from 4 mW of squeezed light excitation is also shown
in the graph where for a coherent light excitation of the same power, the fluorescence is completely unmeasurable. More importantly, matching the fluorescence signal from 8 mW of squeezed light with the CW laser requires at least a power of 55 mW, thus approximately 7 times more intensity.

Given that TPA is highly sensitive to the instantaneous arrival of two photos at the sample, an investigation of the effect of relative temporal delay between the entangled photon pairs on the enhancement of TPA was conducted. Since the entangled two-photon absorption cross-section is inversely proportional to the mean group velocity delay between the entangled pairs (i.e., the entanglement time $T_e$) \[11, 13, 16\], adjusting $T_e$ should change the enhancement accordingly. In the FWM process of the atomic $^{85}$Rb vapor, the group delay between the entangled pairs can be adjusted by changing the two-photon detuning of the double-Λ configuration in the atomic level structure, denoted in Fig. 1(b) as $\delta$ \[24\]. In Fig. 2(b), the fluorescence signal induced by 8 mW of squeezed light with three different $\delta$’s was plotted. The three $\delta$’s have the same relative intensity-difference squeezing of -6.5 dB (see Fig. 1(a)), so as to not have different entanglement levels affect the results. The red, green and blue bars represent the fluorescence signal for $\delta = -10$ MHz, -5 MHz and 0 MHz, respectively. The greater fluorescence signal for $\delta = -5$ MHz as compared to the fluorescence of other detuning values confirms that the enhancement effect is degraded when the relative delay between the photon pairs is tuned away from its optimal position. Experimental details for changing the two-photon detuning can be found in Methods.

In addition, with means of validating the observation of TPA in our optical apparatus, a characterization of the nonlinearity was conducted by obtaining results with different coherent light excitation powers for DCM dyes (see Methods for its preparation). DCM dyes are known to have adequate two-photon absorption properties around excitation wavelength of 800 nm \[11, 25, 26\]. Logically, like in the case of fluorescein, this investigation should yield a quadratic coherent excitation power to fluorescence signal relationship as well. Indeed, as can be observed from Fig. 3 with red squares, the fit is undeniably quadratic. More importantly though, when the DCM dyes are excited with different powers of squeezed light, the excitation power to fluorescence signal relationship has a nonlinear behavior. Indeed, ETPA can be accompanied by nonentangled photons or random classical two-photon absorption effects \[27\]. Therefore, the overall two-photon absorption rate, $R_e$, can be expressed \[14, 16, 28\] as the summation of the linear entangled two-photon absorption rate
FIG. 3. Fluorescence signal of DCM versus excitation power of coherent light (red) and squeezed light (green). Coherent light fitting curve obeys a quadratic behavior, while squeezed light fitting curve shows a polynomial behavior, indicating a high input of entangled photon pair flux. Inset: raw fluorescence signals from PMT for coherent light (red) and squeezed light (green) excitations with 130 µW optical power. Shaded area for each curve represents one standard deviation.

and the quadratic classical two-photon absorption rate, \( R_c = \sigma_e \phi + \delta_r \phi^2 \), where \( \sigma_e \) is the entangled two-photon absorption cross section, \( \delta_r \) is the classical two-photon absorption cross section, and \( \phi \) is the input photon flux density of photon pairs. When the input photon flux is low, the linear term dominates [11–13], while both contributions are significant when the input flux is high. Thus, the polynomial behavior observed for squeezed light excitation in Fig. 3 implies that the input entangled photon pair flux is virtually high enough to render both linear and nonlinear contributions significant.

It is worthy to point out that for a comparable amount of fluorescence signal DCM dyes require much lower excitation power due to a greater classical two-photon absorption cross section \( \delta_r \) as compared to fluorescein. As can be seen from Figs. 2(a) and 3, the fluorescence signal induced by 130 µW of coherent light from DCM is greater than that induced by 8 mW of coherent light from fluorescein. However, the entangled two-photon absorption cross section \( \sigma_e \) of DCM is actually smaller than that of fluorescein, as demonstrated by the fluorescence enhancements. This inconsistency can be attributed to different electronic level
structures of these two organic molecules [13].

In conclusion, this work explores using a squeezed light source for TPA of fluorescein biomarkers. The experimental results demonstrate that as compared to 8 mW of CW coherent light excitation, 8 mW of squeezed light achieves a $\sim 50$-fold TPA fluorescence enhancement. In addition, and differently from previous works using quantum states of light for TPA, we report that SL-TPA is governed by a nonlinear behavior, which can be entirely attributed to its far greater entangled photon pair flux, as compared to using SPDC sources. Thus, this work demonstrates the quantum squeezed light sources can achieve low-intensity TPA for biosensing and bioimaging, and further leverages highly non-invasive deep tissue \textit{in vivo} studies beyond the capabilities of classical TPA.
Methods

Experimental details. In this work an external cavity diode laser and a tapered amplifier was used as the laser source with a typical linewidth (5 µs) of 100 kHz, to generate a strong (in the range of 400 mW to 800 mW) pump beam near the D1 line of Rb (795 nm). From this, a weak probe beam tuned 3 GHz to the red of the pump was acquired by double-passing an 1.5 GHz acousto-optic modulator (AOM). This results in a very good relative phase stability of the probe with respect to the pump. The pump and probe beams are combined in a Glan-Taylor polarizer and directed at an angle of 0.3 ° to each other into a 12.5 mm long vapor cell filled with isotopically pure $^{85}\text{Rb}$ (see Fig. 1(d)). The cell, with no magnetic shielding, is heated to 112 °C. The windows of the cell are antireflection coated on both faces, resulting in a transmission for the probe beam of ∼ 98 % per window. The pump and probe beams are collimated with waists at the position of the cell center of 700 µm and 400 µm $1/e^2$ radius, respectively.

After the cell, the pump and probe beams are separated by a second polarizer, with $\sim 10^5 : 1$ extinction ratio for the pump. The pump at $\nu_1$ is tuned to a ‘one-photon detuning’ $\Delta$ of 900 MHz to the blue of the $^{85}\text{Rb} \, 5S_{1/2}, F = 2 \rightarrow 5P_{1/2}$, D1 transition (see Fig. 1(b)). The probe at $\nu_p$ is detuned $(3036 + \delta)$ MHz to the red of the pump (where $\delta$ is the ‘two-photon detuning,’ typically a few MHz, which can be adjusted by changing the radio frequency that drives the 1.5 GHz AOM) resulting in an intensity gain on the probe of 4.5. This gain is accompanied by the generation of a ‘conjugate’ beam at $\nu_c$, detuned $(3036 + \delta)$ MHz to the blue of the pump, which has the same polarization as the probe, and propagates at the pump-probe angle on the other side of the pump so that it satisfies the phase-matching condition. After the second polarizer, the probe and conjugate beams are directed into the two ports of a balanced, amplified photodetector with a transimpedance gain of $10^5 \text{ V/A}$ and 94% quantum efficiency at $\lambda = 795$ nm. The output of this photodetector is sent to a radio frequency spectrum analyzer with a resolution bandwidth RBW of 300 kHz and a video bandwidth VBW of 100 Hz.

Squeezing measurements. A typical squeezing spectrum is shown in Fig. 1(a). The standard quantum limit (blue curve) of this system was measured by picking off the probe before the cell, splitting it with a 50/50 non-polarizing beam splitter, and directing the resulting beams into the balanced, amplified photodetector. The balanced detection technique subtracts away common-mode noise to better than 25 dB. The balanced photodetector noise
level is a measure of the standard quantum limit for the total amount of optical power arriving at the photodetector. The standard quantum limit should be independent of frequency, which is indeed the case within the bandwidth of the detection electronics, which begins to drop down above 3 MHz.

Samples preparation. Both of the fluorescein and DCM samples were dissolved in standard solvents at room temperature: 100 \( \mu \text{mol}^{-1} \) of fluorescein in water with pH = 13; and 10 mmol\(^{-1} \) of laser dye 4-dicyanomethylene-2-methyl-6-(p(dimethylamino)styryl)-4H-pyran (DCM) in dimethyl sulphoxide (DMSO).

Data acquisition. Fluorescence signals were collected by a PMT running in CW mode with its photocurrent recorded by an oscilloscope. For each excitation power, all PMT’s raw responses (inverse voltage pulses) in a 210 ms time interval were added together, and 10 sets of time intervals were used to obtain the average and the standard deviation. Examples are shown in the insets of Figs. 2(a) and 3.

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Author Contributions

G.S.A. conceived and developed the idea. T.L. implemented the project from the very beginning to its completion. F.L. and C.A. contributed to solving technical issues of the detection scheme. F.L. also participated in data analysis. All contributed to the writing of the manuscript.

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