SMFI with two photon absorption

J.J. Gomez-Cadenas

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1 Two Photon Absorption

Two-photon absorption (TPA) is the physical process by which two photons of identical or different frequencies are absorbed by a molecule. The energy difference between the involved lower and upper states of the molecule equals the sum of the energies of the two photons absorbed. TPA is a second-order process, typically several orders of magnitude weaker than linear absorption at low light intensities. In TPA the transition rate depends on the square of the light intensity. The non-linear nature of the process can make TPA dominate over linear absorption at high intensities. Two-photon absorption can lead to two-photon-excited fluorescence (TPEF) where the excited state produced by TPA decays by spontaneous emission to a lower energy state (figure**??**.

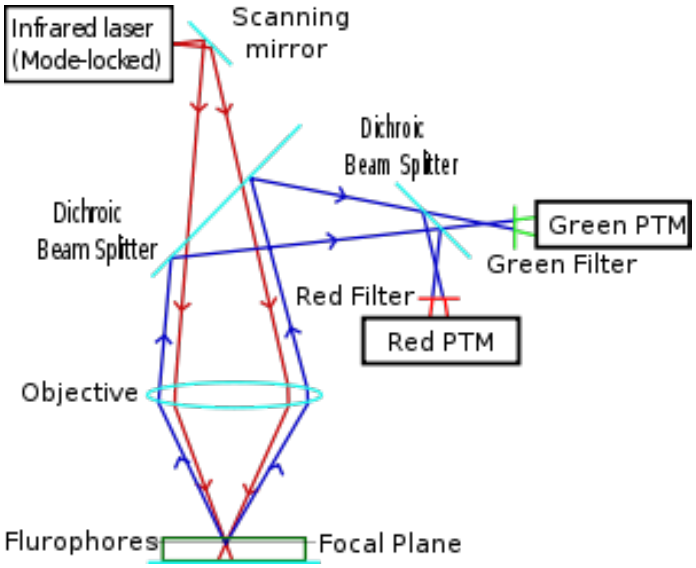

Figure 1: A diagram of a two-photon microscope.

In two-photon excitation microscopy (TPEM) a pulsed laser beam (often infrared, since many common dyes absorb in the blue) is focused through an objective lens. The laser pulse width is in the range of 100 femtoseconds and the repetition rate in the range of 100 MHz, allowing the high photon density and flux required for two photons absorption (figure3).
TPEM may be a good approach to detect SMFI in the mono-layer sensors being planned as detectors of single Ba^{++} ions by the NEXT collaboration.
tagging in NEXT. The projected sensors will be mono-layers located in the detector cathode, thus immersed in a xenon dry atmosphere. This precluded the use of TIRF. In addition, the most suitable sensors may have excitation wavelengths in the UV (*e.g.*near 250 nm). Single photon absorption (SPA) at these frequencies with objectives capable to work in an ultra-pure gas atmosphere (no degassing, no oil) becomes challenging. In addition the optics has to deal with mismatch between excitation light (250 nm) and emission light (425 nm). TPEF has the merit that focusing to sub-micron precision is inherent to the non-linearity of the technique and that the excitation light will be in the range of 500 nm, well matched (but sufficiently separated) from the emission light (425 nm).

2 Mathematical formulation

Because TPA is a second-order process, the number of photons absorbed per molecule per unit time is proportional to the two-photon absorption cross section δ and to the square of the incident intensity I. In a particular experiment, the total number of photons absorbed per unit time N_{abs} is also a function of dye concentration C (assumed to be constant in the absence of saturation and photobleaching) and the illuminated sample volume V. Thus:

$$N_{abs}(t) = C\delta \int_V I^2(\mathbf{r}, t)dV. \tag{2.1}$$

For the lasers used for TPEM, it is possible to separate the time and space dependent of the excitation intensity, $I(\mathbf{r}, t) = I_0(t)S(\mathbf{r})$. Then:

$$N_{abs}(t) = C\delta I_0^2(t) \int_V S^2(\mathbf{r})dV. \tag{2.2}$$

Assuming no stimulated emission and self-quenching, the number of fluorescence photons collected per unit time (F) is given by:

$$F(t) = \frac{1}{2}\epsilon Q N_{abs}(t). \tag{2.3}$$

Where ϵ is the microscope detection efficiency and Q is the quantum efficiency of the dye. The factor 1/2 reflects the fact that two photons are needed for each excitation event.
In practice one measures the time-averaged flux, $< F(t) >$:

$$< F(t) >= \frac{1}{2}\epsilon QC\delta < I_0^2(t) > \int_V S^2(\mathbf{r})dV. \tag{2.4}$$

Notice that $< F(t) >$ is proportional to $< I_0^2(t) >$, but the microscope measures a signal proportional to $< I_0(t) >$. Defining:

$$g = \frac{< I_0^2(t) >}{< I_0(t) >^2}, \tag{2.5}$$

we can write:

$$< F(t) >= \frac{1}{2}\epsilon QC\delta g < I_0^2(t) >^2 \int_V S^2(\mathbf{r})dV. \tag{2.6}$$

2.1 Gaussian laser beam

To compute the integral $\int_V S^2(\mathbf{r})$ it is useful to approximate the laser as a gaussian laser beam, defined as:

$$S(\mathbf{r}) = S(r, z) = \left[\frac{w_0}{w(z)}\right]^2 e^{\frac{-2r^2}{w(z)^2}}. \tag{2.7}$$

The parameter w_0, usually called the Gaussian beam radius (or beam waist), is the radius at which the intensity has decreased to $1/e^2$ or 0.135 of its axial, or peak value. At $2w_0$, or twice the Gaussian radius, the intensity is 0.0003 of its peak value, usually completely negligible.
The evolution with the longitudinal coordinate z of the beam radius is described by the equation:

$$w(z) = w_0\sqrt{1 + (\frac{z}{z_R})^2}. \tag{2.8}$$

where the Rayleigh radius z_R is defined as:

$$z_R = \frac{\pi w_0}{\lambda}, \tag{2.9}$$

and λ is the incident light wavelength.

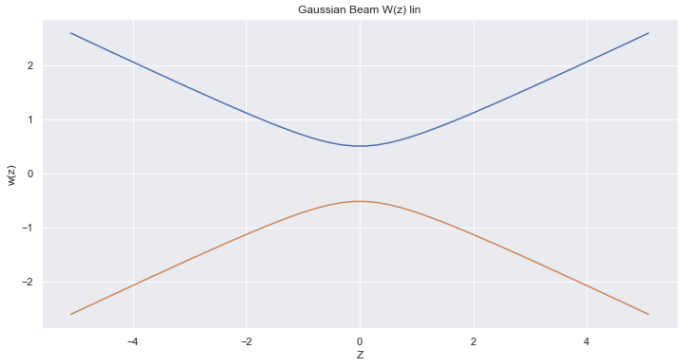

Figure 2: Evolution of the beam radius with the longitudinal coordinate.

The beam power is obtained integrating the intensity distribution:

$$P = \int_0^\infty I(r, z = 0)\, 2\pi r dr = I_0 \int_0^\infty 2\pi r e^{-2r^2/w_0^2} dr = \frac{1}{2} I_0 \pi w_0^2. \tag{2.10}$$

and thus:

$$I_0 = \frac{2P}{\pi w_0^2}. \tag{2.11}$$

The integral of the square of the Gaussian beam to the full volume yields:

$$\int_{V\to\infty} I^2(r, z) = \int_{-\infty}^\infty dz \int_0^\infty I^2(r, z)\, 2\pi r dr. \tag{2.12}$$

The integral in the radial coordinates is:

$$\int_0^\infty I^2(r, z)\, 2\pi r dr = \left[\frac{w_0}{w(z)}\right]^4 \int_0^\infty 2\pi r e^{-4r^2/w_0^2} dr = \left[\frac{w_0}{w(z)}\right]^4 \frac{1}{4}\pi w(z)^2 = \frac{\pi w_0^4}{4w(z)^2} \tag{2.13}$$

The integral in the longitudinal coordinate is:

$$\int_{-\infty}^\infty dz \frac{\pi w_0^4}{4w(z)^2} = \frac{\pi w_0^4}{4} \int_{-\infty}^\infty \frac{dz}{w(z)^2} = \frac{\pi w_0^4}{4} \int_{-\infty}^\infty \frac{dz}{w_0^2(1 + (z/z_R)^2)} = \frac{\pi w_0^2}{4} \pi z_R. \tag{2.14}$$

It follows that for an "infinitely thick sample", we find:

$$\int_{V\to\infty} I^2(r, z) = \frac{\pi w_0^2}{4} \pi \frac{\pi w_0^2}{\lambda} = \frac{\pi^3 w_0^4}{4\lambda} \tag{2.15}$$

Substituting equation 2.15 and equation 2.11 in equation 2.6, we find:

$$< F(t) >= \frac{1}{2}\epsilon QC\delta g \frac{4P^2}{\pi^2 w_0^4}\frac{\pi^3 w_0^4}{4\lambda} = \frac{1}{2}\epsilon QC\delta g \frac{\pi}{\lambda}P(t)^2. \tag{2.16}$$

In the calculation above, the refraction index n has been assumed to be 1. Writing it down explicitly we find the expected value of the TPA induced fluorescence under the approximation of a Gaussian laser beam (notice also that the sample is assume to be infinitely thick compared, since we integrate z between $\pm\infty$):

$$< F(t) >= \frac{1}{2}\epsilon QC\delta g \frac{n\pi}{\lambda}P(t)^2. \tag{2.17}$$

Notice also that, according to equation 2.17, $F(t)$ does not depend on the beam waist (or equivalently of the numerical aperture).

2.2 Temporal dependence for a pulse laser

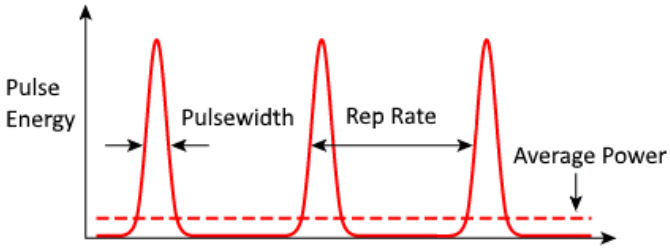

Figure 3: A pulsed beam.

The focused intensity obtained from a mode-locked laser is a periodic function of time defined by the pulse repetition rate (f) and the pulse width (FWHM), τ. For pulses with a temporal profile one can write:

$$I(t) = I_p \exp\left[-4\log 2(\frac{t}{\tau})^2\right] \tag{2.18}$$

Because of the periodic nature of the pulse train, one needs to calculate g for only one cycle.

$$g = \frac{g_p}{f\tau}, \tag{2.19}$$

where:

$$g_p = \frac{\tau\int_{-1/(2f)}^{1/(2f)} I_0^2(t)}{[\tau\int_{-1/(2f)}^{1/(2f)} I_0(t)]^2} \tag{2.20}$$

Substituting 2.18 into 2.21, we find:

$$g_p = \frac{0.75 erf(\frac{2.35}{2f\tau})}{1.134 erf(\frac{1.66}{2f\tau})^2}. \tag{2.21}$$

Setting $f\tau = 1/2$, we find $g_p = 0.664$.

2.3 TPA fluorescence for a gaussian pulsed beam

Substituting 2.21 into 2.17 we find, finally:

$$< F(t) >= \frac{1}{2}\epsilon Q C \delta \frac{g_p}{f\tau}\frac{n\pi}{\lambda}P(t)^2. \tag{2.22}$$

Notice that the units are photons per unit time, as expected:

$$[< F(t) >] = \frac{\text{molecule}}{\text{L}^3}\frac{\text{L}^4\text{T}}{\text{photon molecule}}\text{L}^{-1}\frac{\text{photons}^2}{\text{T}^2} = \frac{\text{photon}}{\text{T}} \tag{2.23}$$

3 Experiment

We will use a pulsed beam with the following parameters:

1. $\lambda = 800$ nm.
2. power (through the objective): 350 mW.
3. $f = 76$ MHz.
4. $\tau = 400$ fs.

With the above parameters the beam illuminates the sample with 1.4×10^{18} photons/second.
The microscope has the following parameters:

1. $NA = 0.5$.
2. $M = 20$.
3. $epsilon = 0.10$

where ϵ is an estimation of the combined efficiency of the optical system and the PMT.
Assuming that our beam has a Gaussian profile and diffraction-limited operation (*e.g.* beam expanded to fill the back of the objective lens), we find:

1. $w_0 = 0.5\ \mu$.
2. $z_R = 1.0\ \mu$.

Which means that the beam penetrates about 1 μ in the radial coordinate and 2 μ in Z. This defines a cylindrical FOV of 1.7×10^{-9} mm^3. The photon density in the FOV area is 1.7×10^{26}photons/(second cm^2). The photon density per pulse is 4.3×10^{29}photons/(second cm^2).
If a sample of 1 nM of molecules (6×10^{11} molecules of Fluo-3) is prepared, we expect 1 molecule per FOV, which would be ideal to observe SMFI.
The molecule is Fluo-3 (figure 4). The cross section for TPA of Fluoresceing (the fluophore in Fluo-3) has been measured[1] and is shown in figure 5. At 800 nm the cross section is of the order of 40×10^{-50}cm^{-4} s/(photon molecule). The quantum efficiency of Fluorescein is about 0.9.

[1] https://www.osapublishing.org/DirectPDFAccess/6D7E7982-90AC-2FC4-1BC45ECB85CF58B0_33721/josab-13-3-481.pdf?da=1&id=33721&seq=0&mobile=no

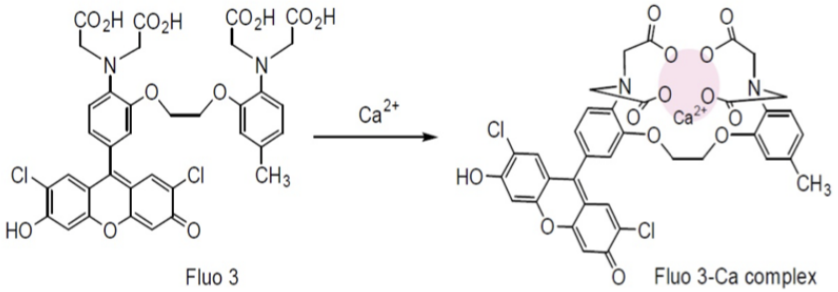

Figure 4: Fluo-3.

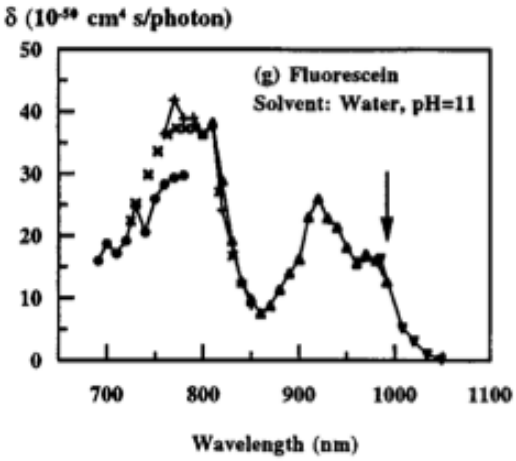

Figure 5: Fluorescein TPA cross section.

Using formula 2.23 we find a fluorescence of 2.2×10^7 photons/second in the absence of photobleaching. Notice that the very small value of the cross section is compensated with the square of the power ($\sim 10^{36}$ photons, the concentration (at least 6×10^{11} molecules/cm^3), the wavelength (1.2×10^4 cm^{-1}, and the term ($f\tau = 2.1 \times 10^4$).
On the other hand, photobleaching may occur (in an oxygenated environment) after the emission of about 36,000 photons[2]. In this case the fluorescence would last 1.7 ms, and 22 photons/μs would be emitted. Assuming an overall detection efficiency of about 10–15 %, one would record about 3 photons/μs. Thus, with a sampling frequency of 20 kHz, one could follow the trajectory of a molecule with about 34 samples and about 150 photons per sample.

4 Methodology

- Prepare several samples: ideally: 1 nM (6×10^{11}), 10 nM and 100 nM of chelated molecules.
- Prepare a control sample: 100 nM (6×10^{13}), unchelated molecules.

[2] https://www.microscopyu.com/techniques/fluorescence/introduction-to-fluorescence-microscopy

- We don't need a full raster scan. Rather the strategy is to focus the microscope in one point, take data and record at 20 kHz, and try to observe SMFI, including a full trajectory until photo-bleaching.