

# Interpretation of Measurements of Photochemical Reactions in Focused Laser Beams

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*Abstract:* - A method is described for obtaining the rate constant of the photodegradation process of fluorophores illuminated by focused laser beam. The explicit kinetic equations are averaged over the illuminated volume to describe the behavior of fluorescence for illumination modulated at low frequencies (1 Hz to 100 Hz). Solution of the kinetic equation for a complex modulation leads to an in-phase and quadrature components. The ratio of quadrature to in-phase components yields information on the photodegradation rate. Modeling is undertaken to explore the accuracy of the measurement of rate constants using the power modulation and synchronous detection technique.

*Key-Words:* - photochemical reactions, photodegradation, fluorescence, frequency domain

## 1 Introduction

There are many instances of a laser beam interacting with biological system[1]. Examples range from laser surgery to simple probing of labeled antibodies. In most cases the interpretation of the measurements is rather difficult. This paper describes a technique which may provide a quantitative method for studying laser induced effects. The work focuses on the photodegradation of fluorescent labels in solution illuminated by a focused probe beam. Fluorescent labels are widely used to probe cell structure and cellular events, and photodegradation is often a limiting factor. There are many difficulties in interpreting the reduction of fluorescence signal due to photodegradation. Heating, convection, and scattering are some of the confounding factors. We describe a method to obtain quantitative information about the photodegradation rate constant of fluorophores. The methodology could be applied to other cases such as photodynamic therapy[2].

## 2 Problem Formulation

Figure 1 shows a schematic of the physical situation under consideration in this work.

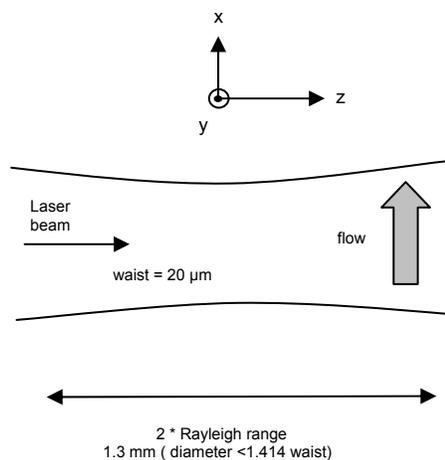


Figure 1

A more detailed description of the apparatus is given in reference [3]. A laser beam, incident along the z axis, is focused to a waist of about 20 μm. A solution of fluorophores flows along the x axis (vertical direction in Fig. 1) and passes through the focused laser beam. A detector located along the y axis (out of the plane of Fig. 1) detects fluorescence from the illuminated fluorophores within the acceptance aperture which is no larger than twice the Rayleigh range (the distance over which the beam diameter does not exceed 1.414 of the value of the waist). The detector sums all photons coming from the fluorophores in the observation region. The kinetic equation that describes the temporal evolution of the fluorophore concentration integrated over the entire observation region,  $\langle N(t) \rangle$ , has been derived [4]. The resulting equation is reproduced below.

$$\frac{d}{dt} \langle N(t) \rangle = \frac{-\eta \alpha (P_0 + \Delta(t))}{1 + b \alpha (P_0 + \Delta(t))} \langle N(t) \rangle - \frac{v}{1.274 \cdot w} \cdot \frac{(1 + b \alpha' (P_0 + \Delta(t)))}{(1 + b \alpha (P_0 + \Delta(t)))} (\langle N(t) \rangle - N^0) \quad (1)$$

where  $\Delta(t) = P_1 \cos(\omega t)$ . Here  $P_1$  is the amplitude of modulated laser power,  $P_0$  is the constant laser power, and  $\omega = 2\pi f$ , the frequency of modulation. The other terms in Eq. 1 are  $v$ , the velocity of flow,  $w$ , the width of the laser beam,  $b$ , a ratio of optical rates involved in the molecular transitions, the unperturbed fluorophore concentration  $N^0$ , and  $\eta = k_d b$  where  $k_d$  is the photodegradation rate. The quantities  $\alpha$  and  $\alpha'$  depend on the beam geometry which is assumed to be a Gaussian function (see Eq. 1c) with the area normalized to 1. Explicitly

$$\alpha \equiv \frac{\int_R f(x, y) f(x, y) N(x, y) dx dy}{\int_R f(x, y) N(x, y) dx dy} \quad (1b)$$

It was shown in a previous paper [4] that to a very good approximation the values of  $\alpha$  and  $\alpha'$  are time independent and that they can be calculated using the time independent, steady state solution of the complete set of kinetic

equations. Consequently we place  $N(x, y)$ , in Eq. 1b. The value of  $\alpha'$  is calculated using Eq 1b with  $N(x, y)$  replaced by the derivative  $dN(x, y)/dx$ . The spatial properties of the laser beam are described by the function [5]

$$f(x, y) = \frac{1}{\pi w^2} \exp\left(-\frac{x^2 + y^2}{w^2}\right) \\ P_0(x, y) = P_0 f(x, y) \quad (1c) \\ \Delta P(x, y, t) = P_1 f(x, y) \cos(\omega t)$$

The fluorescence signal originates from the radiative relaxation of molecules excited by the laser beam. The fluorescence signal from any location within the laser beam depends on the laser power and the concentration of fluorophores at that location. The explicit form of the fluorescence signal integrated over the entire observation volume is given by Eq 2[4].

$$F(t) = B k_{rad} b [P(t) \langle N(t) \rangle - b \alpha (P(t))^2 \langle N(t) \rangle] \quad (2)$$

where  $B$  is an instrument constant,  $k_{rad}$  is the radiative decay rate of the molecular excited state and  $P(t) = P_0 + P_1 \cos(\omega t) \equiv P_0 + \Delta(t)$ . The fluorescent signal can therefore be obtained by solving a single ordinary differential equation for  $\langle N(t) \rangle$ , Eq. 1, and then using Eq. 2 to calculate the resulting fluorescence signal. Together these equations represent an experimentally accessible mathematical model of the fluorescent signal due to excitation by a focused laser beam. The model can be used to analyze measurements. The problem is that the two equations contain many parameters,  $\eta, b, \alpha, \alpha', v$ , and  $w$ , and it is not clear how to extract the value of the relevant physical quantity,  $k_d$ .

### 3 Problem Solution

Consider the case of small laser power. We make the approximation,  $b \alpha P(t) \ll 1, b \alpha' P(t) \ll 1$ , and write Eq. 1 as

$$\frac{d}{dt}\langle N(t) \rangle + (\eta\alpha(P_0 + P_1 e^{j\omega t}) + \frac{v}{1.274 \cdot w})\langle N(t) \rangle = \frac{v}{1.274 \cdot w} \cdot N^0 \quad (3)$$

where we have written the modulated laser power as a complex quantity  $P(t) = P_0 + P_1 e^{j\omega t}$ . By writing the time-dependent power modulation as  $P_1 e^{j\omega t}$  ( $P_1$  real), and assuming a complex solution, we are solving the complex kinetic equation simultaneously for two power modulations:  $P_1 \cos(\omega t)$  and  $P_1 \sin(\omega t)$ . In the case where the response follows the sinusoidal modulation without any lag, the solution will be given by  $\text{Re}^{j\omega t}$  where R is a real number. If the response of the system lags the modulation then there will be a measurable phase lag between the sinusoidal modulation and the response. In this case the solution will be given by  $\text{Re}^{j\omega t}$  where R is a complex number. The complex number R can be written as  $|R|e^{j\theta}$  where  $|R|$  is the magnitude and  $\theta$  is the phase difference between the driving modulation and the complex solutions. The phase is calculated using  $\theta = \tan^{-1}(\frac{\text{Im}(R)}{\text{Re}(R)})$  which, for small phases, reduces to  $\theta = \text{Im}(R)/\text{Re}(R)$  (< 4% error for  $\theta < 17^\circ$ ). Since the phase lag is given by the ratio of the imaginary and real parts of the solution, the calculation of the phase is very simple. The mathematical economy of this method is the motivation for using a complex modulation. Since the modulation contains a constant component,  $P_0$ , the solution is assumed to have a constant component,  $N_0$ , and a time-dependent response,  $N_1$ . The explicit solution is given by Eq. 4.

$$\langle N(t) \rangle = N_0 + N_1 e^{j\omega t} \quad (4)$$

The procedure for finding the solution is to insert Eq. 4 into Eq. 3 and set equal the parts on each side that have the same time variation. (Only the response with the same frequency will be found.

Second harmonic response would require putting in an additional term in the assumed solution proportionate to  $e^{j2\omega t}$ . The time independent part and the part that varies as  $e^{j\omega t}$  are given by Eq. 5

$$\begin{aligned} N_0 &= \frac{A}{A + \eta\alpha P_0} \\ N_1 &= -\frac{\eta\alpha N_0}{A + \eta\alpha P + j\omega} \end{aligned} \quad (5)$$

The constant  $A$  is defined as  $A = v/1.274w$ . According to Eq. 2, for small power levels the integrated fluorescence signal is proportionate to  $P(t)\langle N(t) \rangle$ . Inserting the expression for power of the laser,  $P(t)$ , and the solution  $\langle N(t) \rangle$  given by Eq 5 yields the ratio of the imaginary and real part of the fluorescence signal given by Eq. 6.

$$\frac{\text{imaginary}}{\text{real}} = \frac{\eta\alpha\omega P_0}{A(A + \eta\alpha P_0) + \omega^2} \quad (6)$$

Eq. 6 suggests a possible interpretation of the measurements. Measure the ratio of the imaginary and real parts as a function of frequency for several power levels  $P_0$ . At each power level, fit the frequency dependence to a function of the form  $a_1\omega/(a_2 + \omega^2)$  and obtain the value of the parameter  $a_1$ . According to Eq. 6, the parameter value should depend linearly on power with the slope equal to  $\eta\alpha = k_d b\alpha$ . From the slope it is possible to determine the product of the decay rate, molecular rate constants, and a property of the laser beam. This is not very satisfactory because the result does not provide unique information about the photodegradation rate.

Consider next the case of an arbitrary power level. Repeating the steps leading to Eq. 5 and using the complete Eq. 1 leads to the following solution.

$$N_0 = \frac{A(1+b\alpha'P_0)}{\eta\alpha P_0 + A(1+b\alpha'P_0)} N^0$$

$$N_1 = \frac{-\eta\alpha \frac{1}{(1+b\alpha P_0)(1+b\alpha'P_0)} N_0 P_0}{\frac{\eta\alpha P_0 + A(1+b\alpha'P_0)}{(1+b\alpha P_0)} + j\omega} \quad (7)$$

Applying this solution to calculate the fluorescence signal, we obtain the ratio of the imaginary part and the real part given by Eq. 8

$$\frac{\text{imaginary}}{\text{real}} = \frac{\frac{\eta\alpha P_0}{(1+b\alpha P_0)(1+b\alpha'P_0)} \omega}{\frac{\eta\alpha P_0 + A(1+b\alpha'P_0)}{(1+b\alpha P_0)} A(1+b\alpha'P_0) + \omega^2} \quad (8)$$

At first glance this result does not look promising. However, the denominator is, to a good approximation, dominated by the term  $A^2 + \omega^2$  and the ratio of  $\alpha$  and  $\alpha'$  is approximately a constant for a given laser beam geometry. This ratio can be calculated by Eq. 1b.

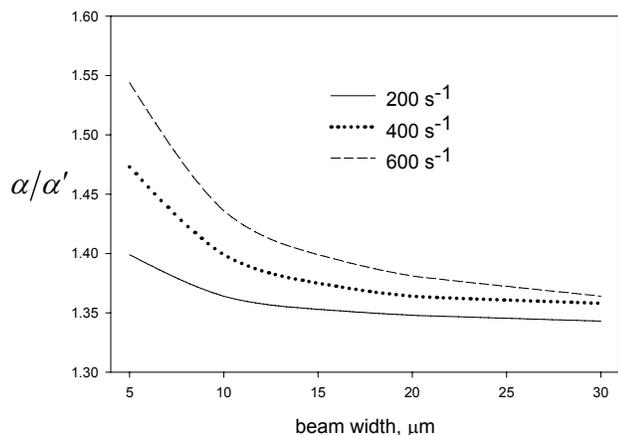


Figure 2

Figure 2 shows the ratio of  $\alpha$  and  $\alpha'$  as a function of beam width for three values of the photodegradation rate,  $k_d$ . In the first approximation, the quantities  $\alpha$  and  $\alpha'$  are related to each other by a constant factor of about 1.40. For specific beam width of 10  $\mu\text{m}$  the value of

the ratio ranges from 1.37 to 1.44 depending on the value of the photodegradation rate constant. In absolute terms, a range of 200 % variation in the photodegradation rate constant results in a 5 % variation in the ratio  $\alpha/\alpha'$ . Thus it is reasonable to set the value of the ratio to a constant in Eq. 8 and proceed to evaluate the photodegradation rate constant. This is demonstrated in the following.

Using Eq. 8 it is possible to calculate the expected response as shown in Fig. 3 for four values of the total laser power.

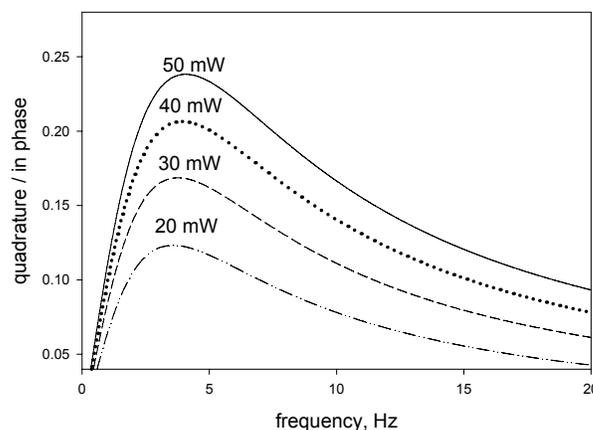


Figure 3

Suppose that the calculated curves in Fig. 3 are actual measurements. The ratio of the quadrature to the in-phase components of the measured response can be fitted to a function of modulation frequency of the form

$$\frac{\text{imaginary}}{\text{real}} = \frac{a_1 \omega}{a_2 + \omega^2} \quad (9)$$

Measurements carried out at different values of power provide a dependence of the parameter  $a_1$  on laser power. Equation 8 suggests that the measured parameter  $a_1$  obtained from the frequency dependence of the response at a given power should depend quadratically on the power. Hence it should be possible to extract two parameters from the fit of the dependence of  $a_1$

on power. The explicit functional form is given by

$$a_1 = \frac{cP_0}{(1+dP_0)(1+d1.44P_0)} \quad (10)$$

The two parameters are  $c=\eta\alpha$ , as in the case of low power, and the additional parameter  $d=b\alpha$ . The ratio of these two parameters gives the photodegradation rate. What is the origin of this happy fact? The variation of  $a_1$  on laser power has two sources, first a variation due to the photodegradation process described by parameter  $c$ , and second variation due to the saturation of the excited state population described by parameter  $d$ . The reduction due to photodegradation involves the same molecular optical parameters as the reduction due to saturation. Therefore in the ratio of the two fit parameters,  $c/d$ , the optical parameters cancel out leaving only the photodegradation rate. The responses shown in Fig. 3 for  $k_d=600 \text{ s}^{-1}$  and different power levels, were fitted and the parameter  $a_1$  obtained as a function of laser power. The upper solid line in Fig. 4 shows the dependence of the parameter  $a_1$  on laser power for  $k_d = 600 \text{ s}^{-1}$ .

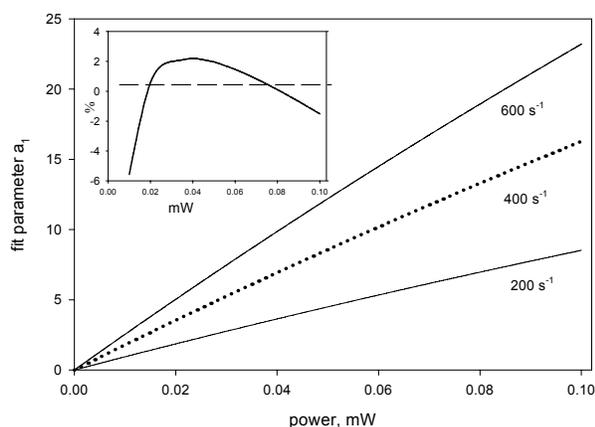


Figure 4

The inset in Fig. 4 shows the deviation of the dependence from a straight line. Although the deviation is small (<5%) it is sufficient to obtain the two parameters  $c$  and  $d$ . The ratio gives a photodegradation constant of  $592 \text{ s}^{-1}$ . The small difference from  $600 \text{ s}^{-1}$  is due to systematic errors inherent in the assumption that the ratio  $\alpha/\alpha'$  is a constant. The present analysis suggests that the systematic errors inherent in the mathematical description are relatively small. In real measurements there would also be additional uncertainties due to the measurement process. The analysis suggests the need for beam stability to maintain a constant beam width. There is a need to limit the detector aperture to accept photons from beam regions over which the beam diameter is maintained to less than 1.414 of its waist.

#### 4 Conclusion

The fluorescence response due to modulation of a laser power can be measured using lock-in amplifier detectors. The ratio of the quadrature (imaginary) to the in-phase (real) components of the measured response can be fitted to a function of the modulation frequency given by  $a_1\omega/(a_2 + \omega^2)$ . The fitting yields values of the parameters  $a_1$  and  $a_2$ . The measurement is repeated for several power levels resulting in a measured dependence of the parameter  $a_1$  on the power of the laser. The dependence of the parameter  $a_1$  on the power should be fitted to a function,  $a_1 = cP_0/((1+dP_0)(1+d1.44P_0))$ , where 1.44 is a property of the laser beam geometry corresponding to a beam width of  $10 \mu\text{m}$ . The ratio of the two fitting parameters,  $c/d$ , gives the photodegradation rate  $k_d$ . In principle the result does not depend on the rates of molecular transitions.

*References:*

- [1] M. H. Niemz, *Laser-Tissue Interactions: Fundamentals and Applications. 3rd edition*, Springer-Verlag, Berlin, **2004**.
- [2] H. Zheng, A Review of Progress in Clinical Photodynamic Therapy, *Technology in Cancer Research and Treatment* **2005**, 4, 283.
- [3] A. K. Gaigalas, L. Wang, R. F. V. Jr., Frequency-domain Measurement of the Photodegradation Process of Fluorescein, *Photochemistry and Photobiology* **2002**, 76, 22.
- [4] A. K. Gaigalas, F. Y. Hunt, L. Wang, Modeling of Photochemical Reactions in a Focused Laser Beam, *Journal of Research of the National Institute of Standards and Technology* **2007**, submitted.
- [5] D. C. O'Shea, *Elements of Modern Optical Design*, John Wiley & Sons, New York, NY, **1985**.