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Optical saturation measurements of fluorophores in solution with pulsed femtosecond excitation and two-dimensional CCD camera detection

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We present a new method for the measurement of saturation of the optical transition of fluorescent molecules in solution, which is based on detection with a CCD camera of a two-dimensional projection of the three-dimensional, spatially nonuniform fluorescence intensity distribution as generated in a bulk solution of the fluorophore by excitation with focused femtosecond optical pulses. Essential to the method is a combination of information from a measurement in saturation and one not in saturation and for the measurement in saturation, the simultaneous observation of both saturated and nonsaturated regions of the fluorescence intensity distribution. The experimental setup is straightforward and good agreement is found between the theory and the experimental data. © 1997 Optical Society of America

Key words: Optical saturation, femtosecond excitation, CCD camera detection.

1. Introduction
As early as 1905, Einstein introduced the phenomenon of saturation of an optical transition. He showed that the degree of optical saturation is dependent on the relative magnitude of the rates of excitation and deexcitation of the excited state. Since these rates depend critically on the detailed energy level structure of a molecule, information about the degree of optical saturation can provide information about photophysical properties of the transition, such as the fluorescence lifetime. Jensen and Schröder used the phenomenon for an extensive optical characterization of laser dyes.

The majority of studies were carried out using fluorescent molecules in the gas phase; see, for example, Refs. 4–11, in which the combination of discrete optical transitions, high absorption cross sections, and relatively slow relaxation pathways makes it relatively easy to achieve saturation of the optical transition. This is much more difficult for fluorescent molecules in solution, in which interactions with the local environment lead to the existence of fast relaxation channels and energy bands rather than discrete energy levels.

To obtain the high excitation intensities needed to achieve saturation of an optical transition, a focused, pulsed laser can be used. Focusing of the laser introduces a spatially nonuniform excitation intensity distribution within the volume under investigation, which, if not properly taken into account, can lead to incorrect estimates for the parameter under investigation. The influence of spatial nonuniformity on the analysis of the experimental data depends on the specific geometry of the experimental setup and is sometimes difficult to correct for. The use of a pulsed laser as an excitation source brings several potential complications with it. For example, the pulsed excitation leads to a pulsed fluorescence emission. Detection of these short fluorescence bursts, with a high peak intensity and a low average intensity, requires a large dynamic range in the detection system. Another complication is that, when the pulse duration is of the order of the time scale of typical energy relaxation processes, all energy levels
involved in the relaxation processes must be considered in the analysis of the data.

Here we propose a new method for measuring saturation of the optical transition of fluorophores in solution, in which the effects of the focused, pulsed excitation are explicitly used in the analysis. The method is based on the illumination of a bulk solution of the fluorophore with focused femtosecond optical pulses at a megahertz repetition rate. The generated spatially nonuniform three-dimensional (3-D) fluorescence intensity distribution is projected onto a two-dimensional (2-D) CCD camera. The use of femtosecond optical pulses with a repetition time much longer than the time scale of typical energy relaxation processes enables a straightforward model for the interaction of the excitation pulse with the fluorescent molecule. The use of a CCD camera in the detection pathway provides, in addition to the required linearity and the large dynamic range, information about the spatially nonuniform distribution of the generated fluorescence. It will be shown that this information can be used to assign a degree of optical saturation to the generated excitation and to construct a 3-D map of the excited-state population density after excitation with the optical pulse.

In Section 2 we give a description of our method to study saturation of the optical transition of fluorescent molecules in solution. In Section 3 we describe the experimental setup for the measurement of optical saturation and present the experimental data. Finally, the experimental results are discussed in Section 4.

2. Theoretical Description

A. Global Description of the Method

In Subsection 2.B we establish the relation between the fluence of an excitation pulse—defined in this study as the number of excitation photons in the pulse per unit area—and the local excited-state population density, generated by excitation with a short optical pulse, using a two-level system approximation. The key element in our approach to optical saturation measurements is the use of a spatially nonuniform excitation fluence distribution, obtained by focusing a laser beam under low numerical aperture conditions. A typical measurement comprises the recording on a cooled CCD camera of the fluorescence generated along the path of propagation of the optical pulses (the optical axis). From the data of such measurements, as detailed in Subsections 2.C and 2.D, the local excited-state population density is derived in two steps. In the first step, Subsection 2.C, we measure the fluorescence distribution at a low excitation fluence, such that the excitation conditions are essentially far from optical saturation. Under these conditions, the excitation fluence distribution can be derived directly from the observed fluorescence distribution. In the second step, Subsection 2.D, we compare the fluorescence in and far from the focal region. Because of optical saturation the fluorescence generated in the focal region, with a high local excitation fluence, shows a smaller increase than the fluorescence in out-of-focus regions, in which, because of the low local excitation fluence, optical saturation effects are absent. In the approach described below, we integrate the generated fluorescence in planes perpendicular to the optical axis and use the variation along the optical axis of this integrated fluorescence as the basis for the analysis. The effect of optical saturation then shows up as a dip in the total fluorescence in the in-focus regions. Without optical saturation no dip is expected. The depth of the dip is a measure of the degree of optical saturation. Combination of the previously determined excitation fluorescence distribution and the dip in the integrated fluorescence makes it possible to derive a 3-D map of the excited-state population density.

B. Energy Level Model and Saturation of Absorption and Emission

In general, fluorescent molecules in solution exhibit a complicated energy level structure. After optical excitation to a vibrational energy state of the first electronically excited state, a number of relaxation pathways is available. These include vibrational relaxation, with a typical time scale of the order of picoseconds,14 fluorescence, which occurs on a nanosecond time scale,14 and a number of other processes such as, e.g., intersystem crossing and fluorescence resonance energy transfer. For excitation with ultrashort optical pulses (pulse duration less than 100 fs), no significant energy relaxation occurs during the excitation pulse. If these femtosecond optical pulses are delivered with a repetition time much longer than the time scale of the energy relaxation processes, the complicated energy level structure can be approximated with the well-known two-level system.15,16 For simplicity we assume that ensemble coherence effects can be neglected so that the two-level system can be described with rate equations rather than optical Bloch equations.16 Following the analysis of Loudon, it can be shown that under these excitation conditions the following relations hold in two-level systems for the fraction of the total population—or the fractional population density—in the ground state and the excited state:

\[ F_1(t) = C \left[ 1 - \frac{C}{C} + \exp(-2BWt) \right], \]

\[ F_2(t) = C \left[ 1 - \exp(-2BWt) \right], \]

(1)

where \( F_1 \) and \( F_2 \) represent the fractional population density in the ground and excited states, \( t \) (in seconds) is the exposure time, \( B \) (in m\(^2\) J\(^-1\) s\(^{-2}\)) is the Einstein coefficient for both absorption and stimulated emission, and \( W \) (in J s m\(^{-3}\)) is the energy density of the excitation field. The proportionality constant \( C \) \((C \approx \frac{1}{2})\) in Eqs. (1) is related to that fraction of the fluorophore population that can be excited under the given excitation polarization conditions. With femtosecond optical pulses no signifi-
cant rotational effects occur during the pulse, so that no effects from such processes are expected.

Since the pulse duration is much shorter than the other time scales in the model, the temporal profile of the excitation pulse and the kinetics of the interaction of the excitation pulse with the two-level system can be neglected. This means that the distribution of the population in the two energy levels is not determined by the time-dependent energy density \( \dot{W} \) but by the total number of excitation photons per unit area or excitation fluence \( D \) (in photons m\(^{-2}\)) in the optical pulse. The expression \( BWt \) in Eqs. (1) can be rewritten in terms of the excitation fluence as follows:

\[
B \int \dot{W} dt = A \sigma D, \tag{2}
\]

where \( \sigma \) (in m\(^2\)) is the absorption cross section of the fluorophore. The factor \( A \) accounts for the relation between the Einstein \( B \) coefficient and the absorption cross section.\(^{16} \) This factor must be determined empirically from the data. With Eq. (2), Eqs. (1) can be rewritten as

\[
F_1(D) = C \left[ \frac{1 - \frac{\sigma}{C} + \exp(-2A \sigma D)}{C} \right], \tag{3}
\]

\[
F_2(D) = C [1 - \exp(-2A \sigma D)]. \tag{5}
\]

For small excitation fluences, the population in the excited state is proportional to the excitation fluence. For higher excitation fluences, the relation becomes nonlinear and shows the occurrence of optical saturation.

After the excitation pulse, no absorption or stimulated emission occurs and the population in the excited state decays to the ground state by way of nonradiative decay or fluorescence. The number of fluorescence photons generated per unit volume \( N_f \) (in photons m\(^{-3}\)) that are due to excitation with an optical pulse with a fluence \( D \) is related to the fractional population density in the excited state through

\[
N_f = n \phi_f F_2(D) = n \phi_f C [1 - \exp(-2A \sigma D)], \tag{4}
\]

where \( n \) (in fluorophores m\(^{-3}\)) represents the number density of the fluorophores and \( \phi_f \) is the fluorescence quantum yield.

C. Determination of the Excitation Fluence Distribution from the Fluorescence Distribution at Low Excitation Fluence Conditions

The geometry of the experimental arrangement is shown in Fig. 1. We assume that the excitation fluence distribution within the cuvette, \( D(\rho, z) \), can be written as a cylindrically symmetric Gaussian\(^{17-19} \):

\[
D(\rho, z) = D_0 \frac{w_0^2}{w^2(z)} \exp\left[ - \frac{2\rho^2}{w^2(z)} \right], \tag{5}
\]

\[
\frac{2N_e}{\pi w_0^2}, \tag{6a}
\]

\[
w^2(z) = w_0^2 \left\{ 1 + \left[ \frac{m^2 \lambda (z - z_0)}{\pi w_0^2} \right]^2 \right\}. \tag{6b}
\]

The radial coordinate \( \rho \) is defined as \( \rho = \sqrt{x^2 + y^2} \). \( D_0 \) is the excitation fluence in the geometric focal point, \( N_e \) (in photons) is the number of excitation photons per pulse, \( \lambda \) (in meters) is the excitation wavelength in the solvent, and \( w(z) \) (in meters) is the beam radius, defined as the half-width of the beam at \( \exp(-2) \) of the maximum value at axial position \( z \). The parameter \( w_0 \) (in meters) is the minimum beam radius, also referred to as the beam waist, which is located at the axial position \( z_0 \). The beam quality factor \( m^2 \) (\( m^2 \approx 1 \)) can be interpreted as the ratio of the observed beam waist and the beam waist that would be obtained if the beam and focusing optics were diffraction-limited. Note that it is not necessary for analysis that the beam and focusing optics be diffraction-limited. The only requirement is for the excitation fluence to be cylindrically symmetric and Gaussian.

The number of fluorescence photons detected with the CCD camera, \( N_f(x, z) \) (in photons m\(^{-2}\)), can be written as the number of fluorescence photons per unit volume in the image space of the projection lens, integrated in the \( y \) direction:

\[
N_f(x, z) = \delta \int_{-\infty}^{\infty} N_f(\rho, z) d\rho,
\]
where $\delta$ is a proportionality constant that relates the fluorescence in the cuvette to that in the image space of the lens. Since the dimensions of the excitation intensity distribution are (much) smaller than the dimensions of the cuvette, the limits of integration can be extended to infinity, thus neglecting the boundaries of the cuvette. In the absence of optical saturation [in the limit of small excitation fluences: $D(p, z) \to 0$], it is possible to find a closed form for $N_f(x, z)$:

$$\lim_{D(p, z) \to 0} N_f(x, z) = 2\delta n A 0 \int_{-\infty}^{\infty} D(p, z) dy = E(z) \exp \left[-\frac{2x^2}{w^2(z)}\right] , \quad (8)$$

where $E(z)$ is a proportionality constant that depends on axial position. This means that, under conditions of a low excitation fluence, the half-width of the profile in the $x$ direction of the fluorescence projected on the CCD camera at a certain axial position $z$ depends only on the distance from the position of the beam waist $z = z_0$, the beam quality factor $m^2$, the beam waist $w_0$, and the excitation wavelength $\lambda$ in the solvent as shown in Eq. (6b). With the excitation wavelength as a known external input parameter and the axial position calculated from the known dimensions of the CCD camera pixels and the magnification of the projection lens, the position of the beam waist, the beam quality factor, and the beam waist are determined in two steps. First, half-width $w(z)$ of the profiles in the $x$ direction at various axial positions $z$ in the CCD image can be determined by fitting these profiles with the Gaussian function in Eq. (8). Second, this experimentally determined relation between $w(z)$ and $z$ is fitted with Eq. (6b). Provided that the excitation fluence distribution can be described as a cylindrically symmetric Gaussian, so that the CCD image can be described with Eq. (8), Eqs. (5) and (6b) show that once the parameters $z_0$, $m^2$ and $w_0$ are determined, this procedure specifies completely the relative distribution of the excitation fluence. Equation (6a) then indicates that combination of this information with the excitation pulse energy completely determines the magnitude of the excitation fluence in any region of the illuminated volume.

D. Determination of the Local Excited-State Population Density from Experimental Data at High Excitation Fluence Conditions

As shown in Eq. (3), the local excited-state population density can be determined by the product of the absorption cross section $\sigma$, the constant $A$, and the local value of the excitation fluence $D(p, z)$. In Subsection 2.C we showed how the distribution of the excitation fluence within the illuminated volume can be derived from experimental data. Therefore, once the product $A\sigma$ is known, the quantity $A\sigma D(p, z)$ can be determined and the local excited-state population density is completely specified. Here we show how to determine the product $A\sigma$ from experimental data. First, we assign a degree of optical saturation $\Gamma$ to the generated excitation. Then $A\sigma$ can be determined from a measurement of the degree of optical saturation as a function of the excitation pulse energy.

We choose the total number of detected fluorescence photons in a plane perpendicular to the $z$ axis, $N_f(z)$ (in photons $m^{-1}$), as the basis for the following analysis. $N_f(z)$ can be obtained by integrating the number of fluorescence photons in the image space of the projection lens over both $x$ and $y$. Using the assumed cylindrical symmetry with respect to the $z$ axis of the excitation fluence distribution, the 2-D integral reduces to a one-dimensional integral:

$$N_f(z) = \delta \int_{0}^{\infty} N_f(p, z) 2\pi dp = 2\pi \delta n A 0 \int_{0}^{\infty} \{1 - \exp[-2A\sigma D(p, z)]\} dp \rho, \quad (9)$$

where again the limit of integration is extended to infinity. A combination of Eqs. (5) and (9) shows that $N_f(z)$ varies nonlinearly with $D_0$ in the focal plane but is a linear function of $D_0$ in regions far from the focal plane, because, in these out-of-focus regions where no optical saturation occurs, the product $2A\sigma D(p, z)$ is much smaller than unity even for high values of $D_0$ (see also Subsection 3.C). In Fig. 2, Eq. (9) is numerically integrated and plotted for two values of $D_0$. At low $D_0$, the number of detected fluorescence photons is independent of the position along the $z$ axis, whereas at high $D_0$ a large relative decrease occurs in the in-focus regions. The occurrence of the relative dip can be understood from the following argument. If no significant absorption of excitation light occurs, the number of excitation pho-
tons in planes perpendicular to the z axis is independent of the position along the z axis. Because \( N_f(p, z) \) is proportional to \( D(p, z) \) at low \( D_0 \), \( N_f(z) \) is also independent of the position along the z axis. At high \( D_0 \), the proportionality holds in the out-of-focus regions, but in the in-focus regions \( N_f(p, z) \) is less than proportional to \( D(p, z) \). Therefore, at high \( D_0 \) a relative decrease of \( N_f(z) \) can be seen in the focal region.

We now define the degree of optical saturation \( \Gamma \) as the depth of the fluorescence dip relative to the fluorescence generated in regions in which no optical saturation occurs:

\[
\Gamma = 1 - \frac{N_f(z_0)}{N_f(|z - z_0| \gg 0)} = 1 - R,
\]

(10)

where \( N_f(z_0) \) and \( N_f(|z - z_0| \gg 0) \), respectively, represent the total number of fluorescence photons in the focal plane (at \( z = z_0 \)) and in a plane in which no optical saturation occurs, i.e., a plane far from the focal plane (at \( |z - z_0| \gg 0 \)). \( R \), defined above as \( N_f(z_0)/N_f(|z - z_0| \gg 0) \), represents the relative focal fluorescence, i.e., the total fluorescence in the focal plane relative to that in a plane far from the focus. With this definition, \( \Gamma \) approaches 0 when \( D_0 \) is small so that the relative focal fluorescence \( R \) is high. As \( D_0 \) increases, the relative focal fluorescence decreases and the degree of optical saturation increases.

The product \( A \sigma \) can be calculated from the degree of optical saturation in two steps. In the first step, the relation between the degree of optical saturation \( \Gamma \) and the excitation fluence in the focal point \( D_0 \) determined from a measurement of the depth of the relative fluorescence dip at various excitation pulse energies. This determination is based on Eq. (10) and a measurement of the excitation fluence (see also the description in Subsection 3.C). In the second step, this experimentally determined relation between \( \Gamma \) and \( D_0 \) is fitted with Eqs. (9) and (10) using the product \( A \sigma \) as the fit parameter. From this fit, the optimum value for \( A \sigma \) is derived. With \( D(p, z) \) and \( A \sigma \) determined, Eqs. (3) shows that, within the assumptions made in the description of the interaction of the excitation pulse with the fluorophore, the local excited-state population density can be calculated anywhere within the illuminated volume.

3. Experiments

A. Experimental Setup and Measurements

The experimental setup is shown in Fig. 3. The excitation source consists of a cavity-dumped mode-locked Ti:sapphire oscillator pumped with a water-cooled argon-ion laser (Innova 310, Coherent, Santa Clara, Calif.). The output of this oscillator consists of optical pulses with a pulse duration of approximately 60 fs and a center wavelength of 800 nm. The repetition rate is user-selectable between 0 and 2 MHz. The pulse energy at 2 MHz is approximately 25 nJ. This beam is frequency doubled in the second-harmonic generation unit (Coherent, Santa Clara, Calif.) with a conversion efficiency of approximately 20%, yielding optical pulses with a peak wavelength of 400 nm and a pulse energy of 5.5 nJ (at 2-MHz repetition rate). The beam from this second-harmonic generation unit was found to be astigmatic. This astigmatism was compensated using a cylindrically deformable mirror M1, with which the separation between the two foci can be controlled. The light was focused with a 38-mm focal length compound camera lens in a quartz cuvette (104FQS, Hellma Worldwide, The Hague, The Netherlands) with a path length of 10 mm, a width of 4 mm, and a height of 40 mm. The fluorescence was imaged onto the CCD camera (Hi-SIS24, Lambert Instruments, Leutingewolde, The Netherlands) through an interference filter with a peak transmission at 503 nm and a FWHM of 10 nm, using a 50-mm focal length compound camera lens. The active area of the CCD camera consists of 768 × 512 pixels of 9 µm × 9 µm each. The sample is a 1.0 × 10^{-6} M solution of Coumarin 152 in methanol. This fluorophore was chosen because its absorption spectrum has a maximum at 400 nm, which coincides with the center wavelength of the light generated in the second-harmonic generation unit. The pulse energy or, equivalently, the excitation fluence actually delivered in the sample can be varied by the insertion of neutral density filters in the excitation pathway. An IBM-compatible PC was used for image collection and exposure control of the CCD camera by way of a mechanical shutter.

B. Determination of the Excitation Fluence Distribution from the Fluorescence Distribution at Low Excitation Fluence Conditions

The first step in calculating the local excited-state population density is the determination of the excitation fluence distribution from a measurement of the generated fluorescence when no optical saturation occurs. Figure 4 represents an image of the generated fluorescence at a low excitation fluence in the
geometric focal point \( D_0 \) as obtained with the CCD camera. At this excitation fluence, no appreciable optical saturation is expected to occur in the illuminated volume, so that the fluorescence distribution is proportional to the excitation fluence distribution. Figure 5 shows the half-width \( w(z) \)—the radius at \( \exp(-2) \) of the maximum—of the profile perpendicular to the optical axis (i.e., along the \( x \) axis; see Fig. 4) of the generated fluorescence projected onto the CCD camera at various positions \( z \) along the optical axis. \( w(z) \) was determined by fitting the profiles to the Gaussian function in Eq. (8). Two of these fits are also shown in the figure. For all fits a correlation coefficient of >0.95 was found. In general, the peak could be described well with the Gaussian, but at the base small deviations occurred (see below). We calibrated the axes of Fig. 5 using the CCD pixel dimensions and magnification \( M \) of the projection lens. This magnification was calculated from the known focal length of the lens and the combined object-to-image distance. Special care was taken to include the effects of refraction at the boundary of the cuvette. It was found that \( M = 5.8 \pm 0.2 \). The error margin was calculated from uncertainties in the object-to-image distance. From the data in Fig. 5, we determined the position of beam waist \( z_0 \), beam quality factor \( m^2 \), and beam waist \( w_0 \) by fitting these data to Eq. (6b). It was found that \( z_0 = 4.62 \pm 0.02 \times 10^{-4} \) m, \( m^2 = 6.2 \pm 0.6 \), and \( w_0 = 6.0 \pm 0.6 \times 10^{-6} \) m. The value for \( m^2 \) indicates that the focus of the beam in the cuvette is not diffraction-limited, which is caused by a combination of small deviations from TEM\(_{00}\) behavior of the beam from the second-harmonic generation unit and lens aberrations. This non-diffraction-limited response is not important for the method as long as the focused beam retains an approximate Gaussian shape. We verified that, despite the non-diffraction-limited focus of the beam, all the profiles perpendicular to the optical axis—two profiles are shown in Fig. 5—could be described well with a Gaussian function. The current data do not allow independent verification of the cylindrical symmetry of the excitation beam. Visual inspection of the beam in the far zone and the entrance pupil of the excitation lens, however, showed no indications of apparent asymmetries (results not shown). This supports the fact that, apart from small deviations (see the insets in Fig. 5), the conditions for the validity of the analysis are fulfilled to within experimental accuracy and the experimentally determined values for \( z_0 \), \( m^2 \), and \( w_0 \) together with the excitation wavelength in the solvent, specify

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Fig. 4. Image of the fluorescence distribution generated with a low \( D_0 \). The exposure time was 20 s. The image was corrected for background contributions by subtracting a so-called dark-field image, which was acquired under the same experimental conditions but without the excitation light present. Indicated are two lines perpendicular to the optical axis, along which the fluorescence is measured for subsequent analysis (see Fig. 5).

Fig. 5. Half-width \( w(z) \) of the profile perpendicular to the optical axis of the generated fluorescence projected on the CCD camera at various positions along the optical axis \( z \). The solid curve represents a fit with Eq. (6b). Insets (a) and (b) show the fluorescence along the lines indicated in Fig. 4. The solid curves in these insets represent fits with a Gaussian function that was used to determine \( w(z) \). The relative error in \( w(z) \) determined in this way was smaller than 5% for all indicated profiles.
completely the relative distribution of the excitation fluence within the cuvette [see Eq. (5)].

C. Determination of the Local Excited-State Population Density from Experimental Data at High Excitation Fluence Conditions

The second step in the analysis involves a comparison between the fluorescence generated at a high and a low local excitation fluence. Figure 6 represents an image of the fluorescence generated with a high excitation fluence in the geometric focal point \( D_0 \). In the focal region, the excitation fluence is sufficiently high to achieve optical saturation. In this region one can observe a relative decrease of the fluorescence with respect to the out-of-focus regions where no optical saturation occurs. In Fig. 7 we plotted the normalized total number of fluorescence photons from planes perpendicular to the \( z \) axis as a function of position along the \( z \) axis for the images at the low and high \( D_0 \) conditions as shown in Figs. 4 and 6. The results in Fig. 7 show the expected behavior as discussed in Subsection 2.D. At high \( D_0 \), a relative dip in the number of detected fluorescence photons is found, whereas no significant dip is visible at low \( D_0 \). The asymmetry in the fluorescence dip at high \( D_0 \) is probably caused by nonoptimal correction for background contributions. From Fig. 7 and using Eq. (10), we calculated a degree of optical saturation of \( \Gamma = 0.7 \) for high \( D_0 \). Under these excitation conditions, the focal region is highly saturated (see below). For low \( D_0 \), the degree of optical saturation is estimated to be \( \Gamma < 0.1 \). At this \( D_0 \), no optical saturation occurs throughout the illuminated volume.

Figure 8 shows the relation between the degree of optical saturation \( \Gamma \) and the excitation fluence in the focal point \( D_0 \) as determined by measuring the depth
of the relation between $N_f$ and the maximum value. This means that, at a degree of excitation fluence, the value of $A\sigma D_0$—and thus the local excited-state population density—can be derived directly from Fig. 8 after the degree of optical saturation at that excitation pulse energy is determined from the CCD image.

4. Discussion

A new method has been introduced for measuring the degree of optical saturation of fluorescence molecules in solution using focused femtosecond laser pulses for excitation and a CCD camera in the detection pathway. A degree of optical saturation can be assigned to the generated excitation directly from an image of the generated fluorescence and a 3-D map of the excited-state population density can be derived from the data.

It is important to emphasize that the theoretical analysis in this paper is valid also in the case of nonoptimal focusing conditions. The only requirement is that the excitation fluence distribution be cylindrically symmetric and Gaussian. The theoretical analysis can be extended to include non-Gaussian and asymmetric excitation fluence distributions, in which case, however, more complete experimental information about the excitation fluence distribution is necessary. This could be accomplished, for example, by supplementing the data set at low excitation fluence conditions with several images acquired perpendicular to the optical axis of the fluorescence generated in a thin fluorescent layer positioned perpendicular to and at various positions along the optical axis.

The experiments described herein were carried out with a laser repetition rate of 2 MHz. This specific repetition rate was chosen because (i) the time between optical pulses is significantly longer than the time scale of typical energy relaxation processes, as required in the theoretical analysis and (ii) at this repetition rate, images with a good signal-to-noise ratio could be acquired within practical exposure times (i.e., less than 1 min). Note that, at this repetition rate, photodegradation (or irreversible photo-bleaching) has a negligible influence on the present measurements. Many thousands of excitation/relaxation cycles are needed to effect significant photo-bleaching of the used fluorophore. During the time interval this would take at a 2-MHz pulse repetition rate—5 to 50 ms—diffusion from the surrounding medium will compensate for the effects of photodegradation. Also, radiationless transitions to the lower-lying metastable triplet state (or intersystem crossing) might occur. From the triplet level, the molecules eventually relax to the ground state, for example, through phosphorescence and oxygen quenching. As the probability for intersystem crossing is much smaller than for fluorescence (especially for laser dyes such as Coumarin 152)\textsuperscript{21} and with typical triplet lifetimes of the order of microseconds, only
a negligible fraction of the total population will accumulate in the triplet level at a 2-MHz pulse repetition rate. Thus intersystem crossing can be expected to have a negligible effect on the population distribution in the energy levels involved in the optical saturation effects studied in our experiments.

A specific aspect of the optical saturation measurements we have shown in this paper is that quantitative information can be obtained about photophysical parameters of fluorescent molecules in solution. The current method can thus serve as a means to study such molecules and their local environment.

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