

# Two-photon interactions at single fluorescent molecule level

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**Abstract.** Single-molecule spectroscopy and single-molecule detection are emerging areas that have many applications when combined with scanning, imaging, and spectroscopy techniques. We have combined a commercial confocal scanning head, to a Ti:sapphire laser and to an inverted microscope, for the detection of single molecule fluorescence of various dyes by two-photon excitation. We collected spot images of fluorescent molecules that have been deposited on a substrate considering both blinking and photobleaching behavior of fluorescent spots. Here, we report data related to two-photon interactions that occur with the following fluorescent molecules: Indo-1, Rhodamine 6G, Fluorescein, and Pyrene. The choice of these specific dyes is based upon their wide use in biological and medical applications together with the varying complexity of their chemical structure that increases from Pyrene to Indo-1. Moreover, we report some data about single molecule studies related to denaturation of an enhanced green fluorescent protein, GFPmut2, under one photon excitation regime, that show a very similar trend to that observed for the already mentioned fluorescent molecules. © 2003 Society of Photo-Optical Instrumentation Engineers.  
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## 1 Introduction

Single-molecule spectroscopy and single-molecule detection are emerging areas that have many applications when combined with scanning, imaging, and spectroscopy techniques.<sup>1–3</sup> An effective system can be based on a fluorescence microscope that is endowed with a three-dimensional (3-D) volume selection ability. A confocal or a multiphoton excitation microscope can be used for 3-D spatial mapping of single fluorescent molecules, based on the ability to selectively collect signals from highly confined volumes.<sup>4,5</sup> To characterize two-photon interactions at the single-molecule level, we collected spot images of fluorescent molecules that have been deposited on a substrate using a two-photon architecture.<sup>6,7</sup> In addition, we included the photobleaching behavior of fluorescent spots. The standard ensemble measurements of the probe in solution yield an average parameter value for a large number of copies of the system of interest. Single molecule measurements do not require ensemble averaging. Within this new scenario, it is necessary to better define the photobleaching process that occurs when the fluorescence that is emitted by a fluorescent molecule drops to zero. When considering single-molecule photobleaching, several definitions can be used.<sup>8</sup> Here, we will consider two of them. The molecule may convert from an excited state, usually with a radiative decay constant that lies in the range of tens of nanoseconds, to a second excited metastable state with a vanishing, radiative constant. Irreversible photobleaching and a related phenomenon called blinking (switching “on” of fluorescence after an “off” interval) are usually attributed to

this type of transition. Another possibility is that the molecule will change its structure so that the molecular ground state assumes a vanishing cross section from the excitation light. This change can be induced by isomerization or thermal absorption. In both cases, the molecular fluorescence emission drops to zero. Here, we report data related to two-photon interactions that occur with the following fluorescent molecules: Indo-1, Rhodamine 6G, Fluorescein, and Pyrene. The choice of these specific dyes is based upon their wide use in biological and medical applications together with the varying complexity of their chemical structure that increases<sup>9</sup> from Pyrene to Indo-1. Moreover, we report some data about single-molecule studies related to denaturation of an enhanced green fluorescent protein, GFPmut2, under one photon excitation regime.<sup>10</sup> For GFPmut2, confocal optical microscopy measurements show a very similar trend to that observed for the already mentioned fluorescent molecules.<sup>11</sup>

## 2 Materials and Methods

### 2.1 Optical Setup

The architecture has been previously described in detail.<sup>6</sup> The laser source is a mode-locked Ti:sapphire laser (Tsunami 3960, Spectra Physics, California) provided by a solid state laser at 532 nm (Millennia V, Spectra Physics, California) that produces 80- to 100-fs pulses at a repetition frequency of 80 MHz with an average power output  $\cong 700$  mW at  $\lambda$

=770 nm. The optical setup was created using an inverted microscope (TE300, Nikon, Florence, Italy) and a confocal laser scanning head (PCM2000, Nikon, Florence, Italy) that was adapted for two-photon excitation. A portion of the laser beam is focused on the entrance of the PCM2000 using a planoconvex lens with a numerical aperture (NA) of 0.15. It then passes through a dichroic mirror (650 DCSRX C72-38; Chroma Inc., Brattleboro, Vermont) and is delivered to the microscope objective (NA=1.4, Plan Apochromat 100X oil, Nikon, Florence, Italy) by the scanning lens. The fluorescence signal, collected using the same objective and selected by a filter (HQ535-50, Chroma Inc., Brattleboro, Vermont), is then fed to a single-mode fiber connected to a photomultiplier (R928, Hamamatsu, Milan, Italy) that is plugged into a PCM2000 electronic controller module. Imaging is performed by the Nikon EZ-2000 software that interfaces the PCM2000 scanning head.

The optical setup for the single-photon imaging experiments is based on a Modular Confocal Microscope System (C1, Nikon, Florence, Italy) mounted on an inverted microscope (TE2000, Nikon, Florence, Italy). The laser beam (argon-ion at 488 nm) is sent to the entrance pupil of the objective (NA=1.4, Plan Apochromat DICH 100 $\times$  oil, working distance 0.19 mm, focal length 2 mm, Nikon) by the scanning lens. The fluorescence signal, collected by the same objective and selected by a filter (HQ515-30, Chroma Inc., Brattleboro, Vermont), is fed to a single-mode fiber connected to a photomultiplier (R928, Hamamatsu, Japan) plugged into the C1 detection box.

## 2.2 Slide Imaging

The point spread function of the imaging system was previously measured<sup>6</sup> and corresponds to a plane resolution of  $\approx 0.21 \mu\text{m}$  and an axial resolution of  $\approx 0.7 \mu\text{m}$ , under the two photon excitation (TPE) regime. Image acquisition (512  $\times$  512 pixels) with residence times in the range 9.6  $\mu\text{s}$  per pixel takes  $\approx 0.24 \text{ s}$ . The field of view employed is in the range 10 to 25  $\mu\text{m}$  and the excitation power is usually  $\approx 5$  to 50 mW after the scanning head lens coupler, unless explicitly stated otherwise. With our measurements at the back focal plane of the microscope objective, the estimated power of the beam that actually strikes the sample is  $\approx 1000$  to 3000  $\text{kW/cm}^2$ . This estimate is based on the transmission data of the microscope objective employed. The slide temperature is maintained at the desired temperature by thermostatic control through a copper ring in contact with the coverslip. Most of the measurements were performed at room temperature,  $T \approx 21.0^\circ\text{C}$ . Two-photon images were taken at  $\lambda = 770 \text{ nm}$  for Rhodamine 6G and Fluorescein, and  $\lambda = 720 \text{ nm}$  for Pyrene and Indo-1. Confocal images of GFPmut2 were taken at  $\lambda = 488 \text{ nm}$ .

## 2.3 Sample Preparation

The Rhodamine 6G solutions were prepared by dissolving Rhodamine 6G (Fluka Chemika, Milan, Italy, Cat. No. 83698) in DMSO at a concentration  $\approx 1 \mu\text{M}$ . The Fluorescein solutions have been prepared by dissolving Fluorescein (Fluka Chemika, Cat. No. 46955) in TRIS buffer at a concentration  $\approx 1 \mu\text{M}$ . The glass slides were spin coated with solutions obtained by diluting the DMSO-Rhodamine and the TRIS-

Fluorescein concentrated stocks in ethanol at concentrations  $\approx 70$  to 300 nM. The Pyrene solutions were prepared by dissolving powder (Fluka Chemika, Cat. No. 82650) in DMSO at a concentration  $\approx 100 \mu\text{M}$ . The Indo-1 solutions were prepared by dissolving Indo-1 powder (Fluka Chemika, Cat. No. 57180) in MilliQ water (Millipore S.p.A., Milan, Italy).

GFPmut2 is a triple mutant Ser65Ala, Val68Leu, and Ser72Ala of green fluorescent protein<sup>7</sup> (GFP). This mutant has a  $\text{pK}_a = 6.46 \pm 0.03$  in the silica gels being used for immobilization.<sup>11</sup> GFPmut2 was diluted in phosphate-containing buffers.

## 2.4 Glass Slide Cleaning

The glass slides were first soaked in a 1% sodium dodecyl-sulfate solution (for 24 h), and then placed in a saturated methanol solution of NaOH (for 2 h). We then employed a two-step procedure to remove the NaOH from the glass. First, we soaked the slides in a 0.1% HCl solution for 2 h and then in diluted chromic solution ( $\text{KCr}_2$  in concentrated phosphoric acid) for an additional 2 h. The glass was then placed in ethanol. Immediately before being spin coated with the fluorescent solution, the glass was thoroughly rinsed with Milli-Q water (Millipore S.p.A., Milan, Italy) and then dried with a nitrogen flow.<sup>8</sup>

## 3 Measurements

Measurements were taken using image analysis. Images were collected using two main procedures: slide imaging, which are static images, and fluorescence kinetic spot imaging, which are a sequential, continuous temporal series of single spots.

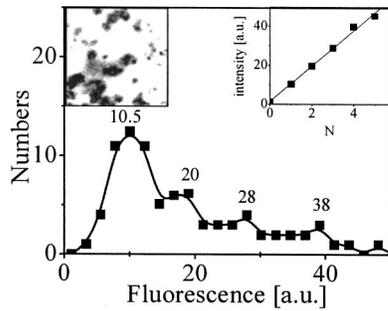
### 3.1 Fluorescence Kinetic Spot Imaging

Sequential images were taken for a total time of  $\approx 100 \text{ s}$ . The fluorescence intensity of each spot was computed by summing the pixel content contained in a circular area located around each spot and by averaging the number of pixels in this area, which typically has a diameter of  $\approx 5$  pixels. The maximum illumination time per spot was found to be  $\approx 1 \text{ ms}$  for a residence time  $\approx 9.6 \mu\text{s}$ . This was estimated considering the time needed to scan an area of  $10 \times 10$  pixels on a  $160 \times 160$  image. The amount of time per image was 229 ms, which corresponds to the time interval that occurs between the subsequent illumination of each molecule.

### 3.2 Optical Setup Check Points

The stability of the microscope stage in the  $z$  direction was assessed by taking a  $140 \times 140 \mu\text{m}^2$  image immediately prior to and after the kinetics on a 10- to 15- $\mu\text{m}$  field of view and by verifying that the only spot missing from the second  $140 \times 140 \mu\text{m}^2$  image was the spot on which the kinetics was performed. In addition, we verified the stability of the acquisition module (Hamamatsu photomultipliers, R928), by utilizing a dc-biased green LED (light emitting diode) and performing long-term kinetics as done for the dye studies. The stability of the signal was found to be  $\approx 0.4\%$  on 100-s kinetics.

The method of computing the total intensity of a spot was carried out by using the home-coded MatLab (Mathworks, Turin, Italy) program LAMBS-IMAGO ver. 7.1. This pro-



**Fig. 1** Upper panel: distribution of the intensity of the spots from a glass slide image prepared by spin coating a  $C=1 \mu\text{M}$  GFPmut2 solution. The scale is in arbitrary units and can be converted into photon counts by multiplying by a factor  $\approx 15$ . Right inset: fluorescence of the peaks in the distribution in order of increasing intensity. Left inset: was acquired with residence time  $\approx 3 \mu\text{s}$ ,  $17 \times 17\text{-}\mu\text{m}$  field of view and excitation power  $\approx 10 \text{ mW}$ . The background level has been acquired from the same image far from any visible spot and is  $\approx 2 \pm 0.15 \text{ au}$ . The numbers on the peaks of the distribution indicate the approximate fluorescence level.

gram also enabled us to identify a single spot on an image time series and to compute the intensity of the spot relative to its acquisition time.

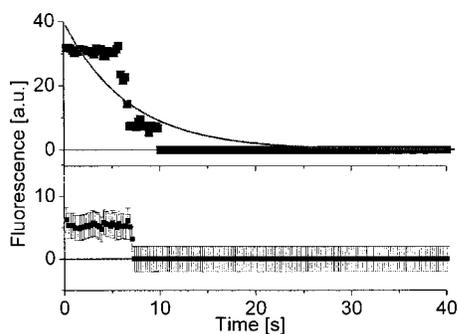
## 4 Results

### 4.1 Fluorescence Static Images

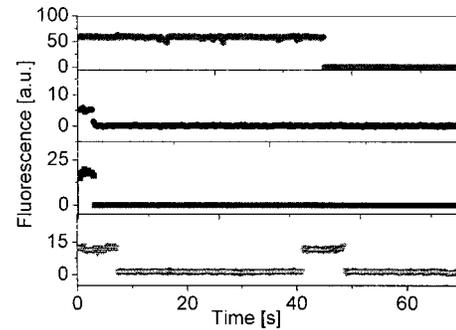
The spatial distribution of the spots is not uniform on the same slide and the brightness can change substantially from spot to spot as shown in the bottom section of Fig. 1. We counted the distribution of the spot intensities. In all cases investigated in this project, the distribution showed discrete peaks and the corresponding fluorescence value scale is linear with respect to the order of the intensity of the spots, as shown in the top inset in Fig. 1. The relative weight of each peak was evaluated by matching the distributions to a sum of multi-Gaussian functions and then plotted in order of increasing intensity, as shown in the bottom section of Fig. 1.

### 4.2 Fluorescence Kinetic Images

We have observed the fluorescence kinetics of several isolated spots on the glass, and discovered varying time evolutions for spots with different levels of intensity. For each spot class



**Fig. 2** Typical Rh6g signal versus time for single molecules (bottom) and for aggregates (top) of the fourth order at  $8.4 \text{ mW}$  of excitation power.



**Fig. 3** Typical fluorescence signal versus time for single molecules of GFPmut2  $\blacklozenge$  (olive), Fluorescein  $\blacktriangledown$  (green), Indo-1 (blu), and Pyrene  $\bullet$  (gray) at  $8.4 \text{ mW}$  of excitation power.

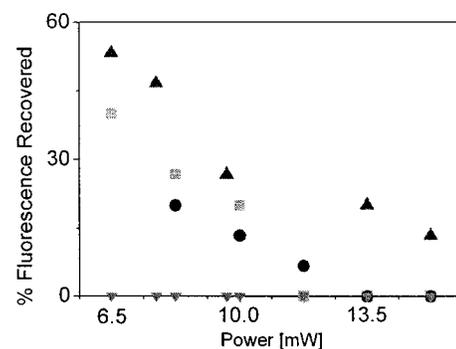
(150 spots), we took the average pixel content as a measure of the total fluorescence emission, subtracting the background value (evaluated using the same image) from the data set. For spots of differing intensity, we observed two different trends that resulted from fluorescence versus time, as shown in Fig. 2. When we study Rhg6, Fluorescein, Indo-1, and Pyrene by means of two-photon excitation we observe an on-off-(on-off) of the fluorescence emitted. A similar on-off fluorescence signal is revealed when we study the GFPmut2 fluorescent protein by means of single-photon excitation.

### 4.3 Recovery of the Fluorescence after Bleaching

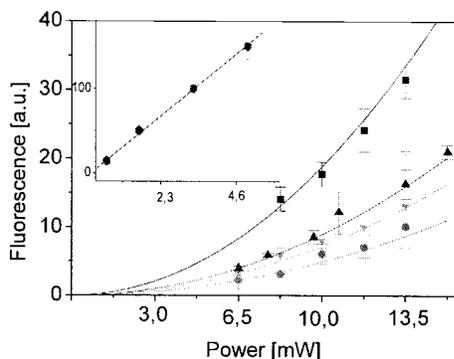
For Rhodamine 6G, Fluorescein, and Indo-1, we found that for a percentage of the single molecules investigated, the fluorescence signal was recovered after the bleaching process, as shown in Fig. 3. For Pyrene and GFPmut2, no postbleaching recovery was observed for the samples and observation times that we employed. For the single molecules, the percentage of the molecules that recovered fluorescence after bleaching strongly depended on the excitation power (see Fig. 4) as well as on the temperature of the substrate.

## 5 Discussion

The intensity of the spots on the glass slides appears in discrete quanta, as shown in Fig. 1. We assigned a number order to each peak of the multi-Gaussian distribution as result of the spots corresponding fluorescence intensity. The peak with the lowest fluorescence intensity was assigned order 1, and the



**Fig. 4** Recovery fluorescence for Indo-1  $\bullet$ , Pyrene  $\blacktriangledown$  Fluorescein  $\square$ , and Rh6g  $\blacktriangle$  versus the excitation power.



**Fig. 5** Fluorescence signal (arbitrary units) for each single molecule versus the excitation power. The average has been computed using at least 100 molecules. The symbols indicate Fluorescein  $\blacktriangledown$ , Rhodamine 6G  $\bullet$ , Pyrene  $\blacktriangle$ , and Indo-1  $\square$ . The data is applied to a law of the type  $A_{\text{EXC}} \times P^2$ . The parameter  $A_{\text{EXC}}$  is reported in the text. Inset: Fluorescence signal (arbitrary units) for single GFPmut2 molecules versus the excitation power upon single photon excitation. The average was computed using at least 100 molecules.

following peaks were assigned with increasing orders: 2, 3, etc. We assume that the average intensity value of the first peak (order 1) corresponds to a single entity, or in other words, to a single molecule. Before investigating fluorescence kinetics, we studied the fluorescence signal trend for each single molecule in relation to the average excitation power,  $\langle P \rangle_{\text{ave}}$ . Under two-photon excitation regime, data were collected between 3 and 50 mW. Figure 5 shows the expected quadratic behavior of fluorescence intensity with respect to average excitation power accordingly with  $(A_{\text{EXC}} \times \langle P \rangle_{\text{ave}}^2)$ . This perfectly agrees to the characteristic law of fluorescence for two-photon excitation, where  $A_{\text{EXC}}$  is a parameter that is connected to the dye cross section.<sup>9</sup> The values of  $A_{\text{EXC}}$  are  $A_{\text{Fluo}} = 0.081$ ,  $A_{\text{Rh6g}} = 0.1$ ,  $A_{\text{Indo-1}} = 0.21$ , and  $A_{\text{Pyrene}} = 0.005$ . These values are in agreement with the two-photon cross section of this dye.<sup>12</sup> We then observed the fluorescence emission of several ( $\cong 100$ ) spots. From confocal optical microscopy measurement of GFPmut2, we observe a linear trend of the fluorescence with the power of excitement:  $(A_{\text{EXC}} \times \text{Power})$  with  $A_{\text{GFPmut2}} = 10$ .

For the spots that were assigned the order 1, the fluorescence remained relatively constant until a sudden drop occurred in a value very close to the background level from the glass slide (top of Fig. 2). When observing the fluorescence kinetics of spots assigned orders  $>1$ , we found a multistep decrease in the signal, as shown in the bottom section of Fig. 2. The number of steps remained consistent with the aggregation order as evaluated from the histogram analysis (for an aggregate of order  $N$  we observed a number of fluorescence drops  $\leq N$ ). Moreover, the fluorescence drop per step was always very close to the quantum of fluorescence per molecule, as measured on the histograms. We considered the on-off trend typical of single molecule fluorescence.<sup>13</sup> In fact, we recorded fluorescence until the single dye entered into a dark state (bleaching). For all molecules, this behavior is related to the number of the molecules that make up the total. This was used as a sort of further criterion to identify single molecules. The time at which the fluorescence of a single molecule falls to zero will vary according to various parameters, namely, (1)

excitation power, (2) substrate temperature, and (3) dye structure. The bleaching time was found to vary in the series Pyrene, Indo-1, Fluorescein, and Rhodamine 6G, from fastest to slowest, respectively. Single molecules of GFPmut2 show a significant stability to illumination with a very long bleaching time.<sup>11</sup> The resulting Gaussian distribution indicated that the behavior observed was not due to irreversible photobleaching, which exhibits an exponential behavior.<sup>14</sup> Moreover, the bleaching time decreased as a function of the glass substrate temperature, reaching a nonmeasurable value for limiting substrate temperatures, whose values changed as found for the bleaching times, from the lowest to the highest temperature, respectively.<sup>9</sup> So far, the observed bleaching showed a strong correlation to both the amount of absorbed power not reirradiated as fluorescence, as well as, in part, to the complexity of the molecule, as it alone could not explain the phenomenon. For Rhodamine 6G, Fluorescein, Pyrene, and Indo-1, these observations were interpreted as thermal bleaching where the temperature increase was induced by the two-photon absorption of the single dyes as confirmed also by numerical simulations.<sup>9</sup> A similar behavior may be responsible for the observed bleaching for the single molecules of GFPmut2 on glasses. However, for a more complete analysis, that should also take into account protein folding and unfolding, we are now developing bleaching measurements with GFPmut2 encapsulated in silica gel.

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