Single-molecule spectroscopy with 27 fs pulses: Time-resolved experiments and direct imaging of orientational distributions

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Confocal microscopy of single molecules bound on a silica surface is performed with precompressed 27 fs laser pulses. Interferometric autocorrelation using a single molecule is demonstrated. It is also shown that orientational distributions can be directly obtained from one- and two-photon images produced with circularly polarized light. © *1998 American Institute of Physics.* [S0003-6951(98)02427-9]

The visualization of processes involving single molecules or assemblies using one-[1–7] or two-photon[8,9] excitation yields knowledge that is not otherwise obtainable. Here we show that one molecule can simultaneously detect two delayed 27 fs pulses and that circularly polarized light[10] can directly image orientational distributions.

The sample of single Rhodamine B (RhB) molecules ($\sim$0.2 molecules per μm^2) was prepared by spin coating a 10^{-10} M solution of RhB in methanol onto a cleaned cover glass. These molecules were each studied in a scanning confocal microscope.[11] For the circularly polarized one-photon experiments we used a mode-locked and frequency doubled yttrium-aluminum-garnet (YAG) laser (wavelength: 532 nm; repetition rate 76 MHz). Several images (integration time per pixel: 2 ms) were recorded using an average excitation power of 0.5 μW. For the two-photon experiments we used a mode-locked Ti:sapphire oscillator (pulsewidth: 20 fs, repetition rate: 80 MHz, pulse spectrum centered at 805 nm). The linearly polarized beam (0.7 mW) was split into two separate beams and one of them passed through a mechanically scanned delay line. After recombination they were made circularly polarized by a broadband $\lambda/4$ waveplate. In both cases the detected count rate of a single dye molecule was obtained by averaging the measured count rate of a circular area with a diameter of 10 pixels, centered at the position of a single molecule spot. For two-photon bulk measurements[12] the induced fluorescence was measured by focusing the linearly or circularly polarized beam (using a $f/10$ cm lens) into a 1-mm-pathlength sample housed in a quartz cuvette. The fluorescence was collected collinear to the excitation path for all but the aluminum phthalocyanine chloride sample, which was collected in a 90° configuration.

We first consider a molecule fixed to a surface (xy-plane) with its unit transition dipole for absorption, $\hat{\mu}$, defined by the colatitude θ and azimuth ϕ. The angular part of the probability absorbing a photon is $|\hat{\mu}\cdot\hat{E}|^2$ where $\hat{E}=\alpha\hat{x}+\beta\hat{y}$ is the unit electric field vector for excitation light propagating along $\hat{z}$, and $\alpha\alpha^*+\beta\beta^*=1$. A microscope objective collects the light from a solid angle less than 2π given by its numerical aperture (NA). The collection efficiency, $\eta(\theta')$, of the emission from one dye molecule depends on the orientation of its dipole moment with respect to the optical axis of the microscope:[13]

$$\eta(\theta')=\tfrac{1}{2}-\tfrac{3}{4}\cos\theta_{\max}+\tfrac{1}{4}\cos^3\theta_{\max}+\tfrac{3}{8}(\cos\theta_{\max}-\cos^3\theta_{\max})\sin^2\theta',$$

where θ' is the colatitude of the transition dipole with respect to that axis and $\mathrm{NA}=n\sin\theta_{\max}$, where n is the index of refraction of the medium between the sample and the objective. Values of $\mathrm{NA}=1.3$ and $n=1.52$ yield $\eta(\theta')\approx 0.146+0.142\sin^2\theta'$. The total detector signal is proportional to $S=\eta(\theta')|\alpha\cos\theta_{x\mu}+\beta\cos\theta_{y\mu}|^2$, where $\theta_{x\mu}$ and $\theta_{y\mu}$ are the angles between $\hat{\mu}$ and $\hat{x}$ and $\hat{y}$. For linear polarization α and β are real and $S=\eta(\theta')\sin^2\theta\sin^2(\phi+x)$ where $x=\tan^{-1}(\alpha/\beta)$. In a fluorescence image of many molecules on an azimuthally isotropic surface the relative intensity of the single molecule signals depends on the three molecular angles θ, θ', and ϕ and the NA. However $S=\eta(\theta')1/2\sin^2\theta$ for circularly polarized light ($\alpha=1/\sqrt{2}$, $\beta=\pm i/\sqrt{2}$), so there is no ϕ dependence. If the same transition dipole is used in the absorption and emission processes ($\theta=\theta'$) and if no angular motion occurs between the absorption and emission steps, the signal probability for circularly polarized light for our microscope is given by $S_1=(0.146+0.142\sin^2\theta)1/2\sin^2\theta$. In this case the distribution of the signals from individual molecules can directly give information on the distribution $P(\theta)$ of θ-angles.

The two-photon induced fluorescence intensity from single molecules, $|EE{:}T|^2$, depends on the structure of the two-photon tensor, T, in the molecular frame, established by the symmetry of the electronic states involved in the transition. Since laser dyes are generally quite polar and have very strong transitions, the two-photon tensors for their emissive transitions are likely to contain only the diagonal element corresponding to the component along the dipolar axis. Choosing $\hat{E}$ as circularly polarized gives a distribution $\eta(\theta')1/4\sin^4\theta$ for the case T has one diagonal element for a molecular axis making an angle θ with the z axis. If the absorption and emission axes are identical this predicts for our microscope a signal $S_2=(0.146+0.142\sin^2\theta)1/4\sin^4\theta$, which also can yield a θ distribution function. Table I gives the expressions for the single molecule absorption for some common two-photon tensors.

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TABLE I. Two-photon fluorescence from single molecules. θ_i and ϕ_i are the colatitude and azimuth respectively of the molecular axis i; x_i is the projection of the molecular axis i onto the arbitrary linear polarization (x) axis, that is $x_i=\sin\theta_i\cos\phi_i$; T_{ij} is the two-photon tensor element; i and j are the principal dielectric axes of a molecule; $\langle\ \rangle$ means ensemble average. The results in this table apply to the two-photon fluorescence induced by two fields having the same frequency and polarization. More information could be obtained by employing two frequencies.

Tensor elements	Linear (single molecule)	⟨Linear⟩	Circular (single molecule)	⟨Circular⟩
T_{11}	$\sin^4\theta_1\cos^4\phi_1 T_{11}^2$	$\frac{1}{5}T_{11}^2$	$\frac{1}{4}\sin^4\theta_1 T_{11}^2$	$\frac{2}{15}T_{11}^2$
T_{11},T_{22},T_{33}	$\sum_i x_i^4 T_{ii}^2+2\sum_{i<j} x_i^2 x_j^2 T_{ii}T_{jj}$	$\frac{1}{5}\left(\sum_i T_{ii}^2+\frac{2}{3}\sum_{i<j} T_{ii}T_{jj}\right)$	$\frac{1}{4}\left(\sum_i \sin^4\theta_i T_{ii}^2+2\sum_{i<j}(\cos^2\theta_i\cos^2\theta_j-\cos^2\theta_k)T_{ii}T_{jj}\right)$	$\frac{2}{15}\left(\sum_i T_{ii}^2-\sum_{i<j} T_{ii}T_{jj}\right)$
T_{12},T_{21}	$x_1^2x_2^2(T_{12}+T_{21})^2$	$\frac{1}{15}(T_{12}+T_{21})^2$	$\frac{1}{4}\sin^2\theta_1\sin^2\theta_2(T_{12}+T_{21})^2$	$\frac{1}{10}(T_{12}+T_{21})^2$

When the two-photon tensor contains more than one element the distribution of intensities now contains information about the θ angles for more than one direction in the molecular frame. This could be extremely useful in establishing the orientation of a single molecule relative to the surface. Circular polarization gives quite a different fluorescence distribution from that induced by two separate fields, one of $\hat{x}$ and one of $\hat{y}$ polarization which, for a single tensor element, yields an absorption part equal to $1/4\sin^4\theta\sin^2(2\phi)$, and thus depends on two angles.

The use of circularly polarized light in imaging has a significant advantage: The intensity distribution from the various single molecule spots only depends on the colatitudes and not on the azimuths of the transition dipoles. A joint distribution for two colatitudinal angles might be obtained for more complex tensors. The two-photon absorption tensor of a particular dye can often be deduced from a bulk measurement.[10]

Dispersion of femtosecond pulses passing through the ~4 cm pathlength of glass in our microscope optics leads to significant pulse broadening and was therefore precompressed by a pair of SF11 glass prisms. An interferometric autocorrelation of the laser pulses was obtained using the two-photon fluorescence of a concentrated RhB sample [Fig. 1(a)]. The field of view of the microscope contained approximately 500 molecules. From a fit assuming a Gaussian intensity profile with zero phase distortion,[14] the full width at half maximum of the intensity envelope of the pulse was determined to be 27 fs at the focal plane of the microscope. The quality of the fit indicates the absence of significant phase distortion over the bandwidth of the pulse. We then used single RhB molecules as the autocorrelating medium for the same femtosecond pulses. A molecule was first located on a confocal image and then zoomed in on. A typical trace of a single-molecule intereferometric autocorrelation is shown in Fig. 1(b). The sudden break-off of the signal is due to photobleaching which typically occurred during the 30 s delay line scan. The photobleached product exhibits a small signal giving rise to a weak interference signal. Our results verify that one can combine femtosecond laser methods with confocal microscopy, which opens the way to carry out two beam experiments with variable delays.

Information on the two-photon absorption tensors of several dye molecules was obtained from bulk two-photon measurements (see Table II). Most dyes showed a ratio near 1.5 for the signals from linear and circular polarization which confirms that they have diagonal tensors with one dominant element[10] (see Table I). In addition we measured this ratio for concentrated RhB samples in the focus of the confocal microscope. The result is in good agreement with the bulk measurement.

An image of single RhB molecules with two-photon excitation is shown in Fig. 2(b). The count rate per pixel ranges from 1 (background) to 29 counts (maximum). The statistical analysis of the integrated counts of 136 single molecule spots is presented in Fig. 3(b). Only isolated round spots were used for the analysis. Figure 3(d) shows the angular distribution calculated from S_2, by assuming that $\theta=\theta'$ and that the largest signal seen is from a molecule with $\theta=\pi/2$. The distribution has a mean of 59° and a standard deviation of 7°, implying that, e.g., due to electrostatic interactions with the surface or the water contamination layer, all the molecules might be forced into a similar tilted position. However, if the brightest molecule does not have $\theta=\pi/2$, the distribution will be even narrower. A previously published measurement[15] on a submonomolecular layer of Rhodamine 6G molecules on a LaSF31 glass surface found $\theta>80°$.

A typical one-photon image is shown in Fig. 2(a) where the count rate per pixel ranges from 6 (background) to 165 counts (maximum). Signals from 700 to 6000 counts per single molecule spot were found from a study of 237 mol-

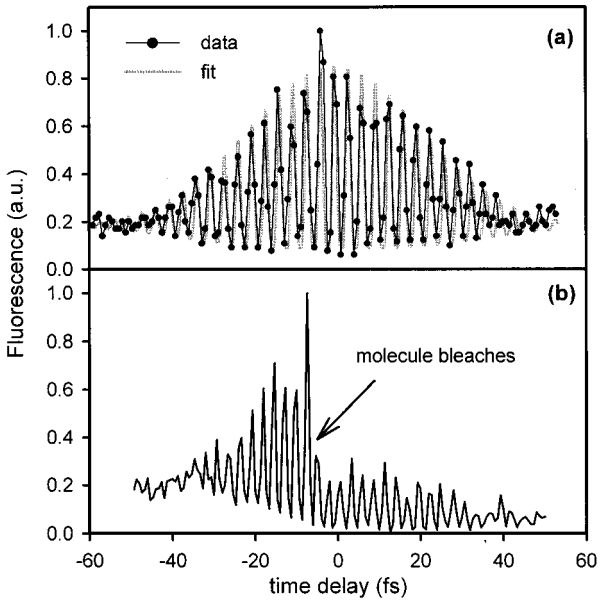

FIG. 1. Autocorrelation of two 27 fs pulses on a sample of ~500 RhB molecules (a) and of a single RhB molecule (b) on a glass surface.

TABLE II. Fluorescence signal of linear excitation divided by circular excitation for different dyes.

Dye	Solvent	Linear/circular
Fluorescein	methanol	166±0.01
Rhodamine B	methanol	1.61±0.02
HPTS[a]	methanol	1.60±0.01
Coumarin 503	ethanol	1.52±0.01
Lucifer Yellow[b]	water	1.33±0.02
AlPc[c]	methanol	1.05±0.01
Rhodamine B[d]	methanol	1.54±0.02

[a]HPTS=8-hydroxypyrene-1,3,6 trisulfonic acid, trisodium salt.
[b]Lucifer Yellow CH, lithium salt (obtained from molecular probes).
[c]AlPc=aluminum phthalocyanine chloride.
[d]Measured in the confocal microscope.

ecules [Fig. 3(a)]. Analogous to the two-photon experiment, Fig. 3(c) shows the angular distribution obtained from S_1. The distribution has a mean of 49° and a standard deviation of 11°. To determine the actual distribution function the orientation of one molecule in each sample must be known. In principle this can be done by recording the fluorescence of a molecule as a function of the numerical aperture.[13] We analyzed the angles θ of 15 molecules by varying the aperture stop of our objective to obtain $0.8 \leqslant \mathrm{NA} \leqslant 1.3$. The results gave an angle $\theta = 20° \pm 20°$, however the method is too insensitive to provide a definitive value of θ.

We considered other effects which could contribute to the distribution of measured intensities from single molecules. One effect arises because a single molecule can undergo reversible transitions to darkstates[2,5] on a time scale from ms to seconds, e.g., due to quenching. This depends on the local environment and might differ from molecule to molecule. Another arises if the molecule can rotate within a fixed solid angle faster than the time resolution of our measurement, the calculated angle distribution would shift to lower values and become narrower. In addition single molecule fluorescence spectra at room temperature show spectral jumps[3,4] on a time scale between 0.1 s and several seconds. If there were fluctuations in the fluorescence Stokes shift, the

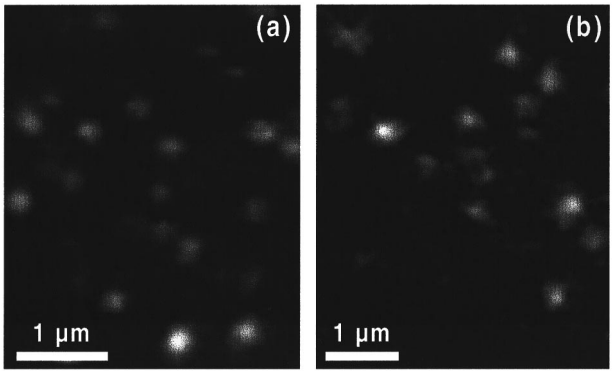

FIG. 2. Fluorescence images of single RhB molecules in circularly polarized light with (a) one- and (b) two-photon excitation.

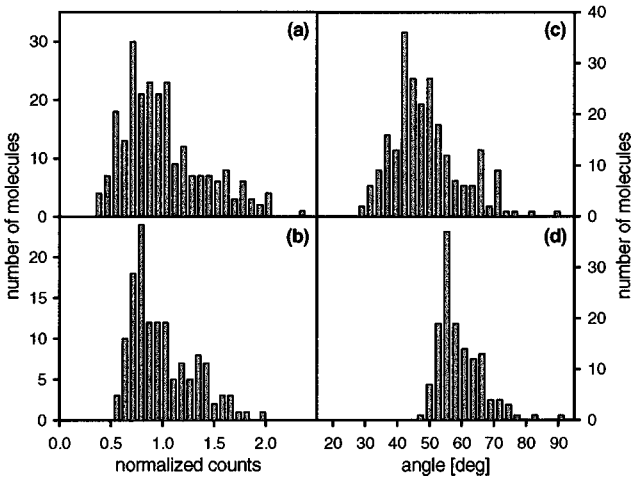

FIG. 3. Statistical analysis of the intensity of single molecule spots studied with one- (a),(c) and two-photon (b),(d) excitation.

detected intensities would not be affected, because of our broadband detection. If there were significant fluctuations in the absorption spectrum of RhB, the observed intensity distribution would be affected, because of the narrow excitation wavelength of our laser. This effect would partly be washed out by the data-acquisition time in our experiment. However, any effect of jumps in the absorption spectra should be drastically reduced in the two-photon experiment, where broadband pulses are used. Figure 3(d) shows that the width of the calculated angle distribution using two-photon excitation is indeed smaller than with one-photon excitation and the mean angle is slightly increased. Alternatively, the measured distribution will yield information about these effects if the orientation of the molecules were known.

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