

Theoretical Investigation of Aspects of Single-Molecule Fluorescence Detection in Microcapillaries

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In the present report, the results of a theoretical investigation of two aspects of single-molecule detection by laser-induced fluorescence in microcapillaries are presented. The two issues studied are the scattering of the exciting laser beam on the microcapillary and the change of the fluorescence lifetime of the molecule due to the electrodynamic interaction between its fluorescence emis-

sion and the confining capillary. Numerical results for experimentally relevant conditions are provided. *Cytometry* 36: 195–199, 1999. © 1999 Wiley-Liss, Inc.

Key terms: single-molecule detection; laser light scattering

Fluorescence detection and spectroscopy of a single molecule in liquids at room temperature has become a routine technique over the past few years (1–3). A crucial point in achieving the single-molecule detection limit is the minimization of any kind of background signal, foremost the rejection of the scattered laser excitation light. This can be achieved by using very small detection volumes. The problem then arises as to how to bring the molecules to be detected into these small volumes. In the simplest setup, based on confocal microscopy, one waits until a molecule is diffusing into the detection volume by chance (4). However, in that case, one is not able to detect a sample of given molecules in a controlled way as needed, for example, in such applications as DNA sequencing (5). More sophisticated setups use hydrodynamic focusing of sample molecules through the detection volume (6,7) or confine the molecules within microdroplets, which can be handled in a much more controlled way (8,9).

Recently, new experiments have been reported concerning the fluorescence detection of single molecules within ultrasmall microcapillaries (inner diameters of a few hundred nanometers) (10,11). One amazing observation was the small intensity of the detected scattered light, defying the usual experience that the light scattering on glass-water interfaces is so great as to prevent efficient single-molecule detection. Thus, one goal of the present study was to present results of a theoretical study concerning the backscattering of a focused laser beam by such microcapillaries.

When detecting single molecules in a microcavity such as a microcapillary with diameters of the order or less than the wavelength of the fluorescence light, one may expect changes in the fluorescence characteristics of the molecule due to an electrodynamic interaction between the

fluorescing molecule and the enclosing cavity. Both theoretical and experimental results have been reported concerning changes of fluorescence lifetime and spectra of molecules confined in spherical microdroplets (12–14). In the present report, I present similar theoretical results for the fluorescence of single molecules confined in a capillary cavity.

The report is organized as follows. In the next section, I present a short overview of the theoretical approach used in answering the above-stated questions. In the third section, numerical results for experimentally interesting configurations are presented.

THEORETICAL BACKGROUND

The studied experimental setup is depicted in Figure 1. A laser beam is incident on a capillary orthogonal to the axis of the capillary. The fluorescence is detected in epifluorescence mode, i.e., from the direction of the incident laser beam. Thus, we will be mainly interested of the backscattering intensity. The calculation of this intensity is done in the following way. The laser beam (Gaussian mode) is thought of as a superposition of plane waves; for each of these waves, the scattering on the capillary can be calculated analytically; the final answer for the whole beam scattering is given as a resummation of the scattering contributions of all the plane waves in the superposition. For the technical details, see Enderlein (15). When calculating the scattering intensities, two basically distinct situations have to be considered: the main polarization of the

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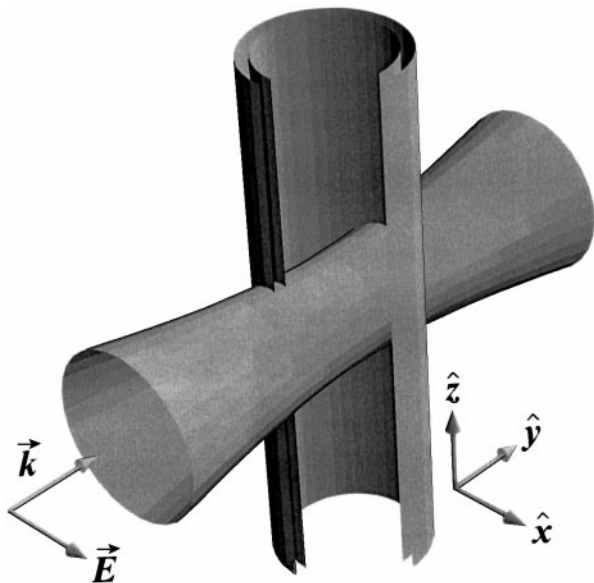


FIG. 1. Geometry of the laser beam scattering on a capillary. The hyperboloid of revolution indicates a surface of constant intensity of an unperturbed incident laser beam with propagation direction \vec{k} and main polarization \vec{E} (transversal to the axis of the capillary).

laser beam is along the axis of the capillary, and the laser beam polarization is transversal to that axis.

For studying the effects of the confining microcapillary on the fluorescence lifetime of the molecule, a semiclassical approach was used, very similar to those of other studies (16–18). The fluorescing molecule is considered an oscillating electric dipole, and the interaction of its electromagnetic field with the capillary is calculated. The resulting electromagnetic back reaction of the capillary on the dipole then yields the change of the emission intensity (thus fluorescence lifetime). This approach is analogous to a quantum-mechanical treatment on the basis of Fermi's golden rule and mode counting of the electromagnetic field mode density of the capillary system (19). Such a simple approach is justified because of the weak coupling of the electromagnetic field of the dipole of the molecule to the capillary. For the technical details, the reader is referred to Enderlein (20).

NUMERICAL RESULTS AND DISCUSSION

First, we studied the backscattering of a laser beam (scattering intensity along the negative y direction in Fig. 1) incident on a glass capillary (refraction index, $n_g = 1.5$) suspended in water (refraction index, $n_{out} = 1.33$) and filled with a water/dye solution (refraction index, $n_{in} = 1.33$). The numerical calculations showed that the scattering intensities for a laser beam with its polarization along the axis of the capillary is much stronger than the scattering for a transversally polarized beam. This is consistent with the well-known fact that elongated wavelength-sized objects scatter light much stronger if the light is polarized along the elongation direction than if the light is polarized transversal to it. Thus, only numerical results for the more interesting case of transversal polarization are

presented. The result is shown in Figure 2, where the backscattering intensity is plotted as a function of the inner and outer diameters, R_1 and R_2 , of the capillary. As can be seen, the scattering intensity has, within the (R_1, R_2) parameter space, an islandlike structure, with islands of maxima divided by bands of minima. Attention should be paid to the large differences between the minimum and maximum values of the scattering intensities. The capillaries used in real experiments are, of course, not ideal cylinders but have a rather conical shape. Thus, the presented numerical results are only an approximation of the experimental situation. However, they suggest that, by scanning the laser beam along the conically shaped capillary, one has a good chance to find a position with values of inner and outer capillary radii that will result in a very low backscattering intensity.

In a real experiment, the observing microscope objective collects not only light scattered back at exactly the negative y direction but also within a cone defined by the numerical aperture of the used objective. The dependence of the detected amount of scattered light on the value of the numerical aperture is shown in Figure 3. There, the inner capillary radius was fixed at $R_1 = \lambda/2$, where λ denotes the vacuum wavelength of the laser light. As can be seen, the positions of the maxima and minima are not changed, and the difference between maximum and minimum intensities of detected scattered light remains significant. This result indicates that the backscattering intensity is indeed a good indicator for finding optimal capillary radii with a minimum of light scattering into the observing objective.

When calculating the change of the fluorescence lifetime, the value of the inner capillary radius R_1 was fixed at $\lambda/2$. As variable parameters, the outer capillary radius R_2 , and the radial distance r of the molecule from the axis of the capillary are considered. Three distinct emission dipole orientations had to be considered: the emission dipole is oriented along a radial line pointing away from the axis of the capillary (Fig. 4), the emission dipole is perpendicular to such a radial line and to the axis of the capillary (Fig. 5), and the emission dipole is oriented along the axis of the capillary (Fig. 6). As can be seen, the most dramatic change occurs for a dipole orientated along a radial line, similar to the case of a vertical dipole over a flat surface.

In a real experiment, the position and orientation of a fluorophore should not be fixed in space. Two different situations may occur: the molecule is freely diffusing within the capillary, or it is adsorbed at the capillary wall. In the first case, the measured fluorescence lifetime is an average of the lifetimes for all possible dipole positions and orientations within the capillary (it is assumed that the measurement time is much larger than the characteristic translational diffusion time and that the fluorescence emission lifetime is much shorter than the characteristic rotational diffusion time). In the second case, the dipole position is fixed at the inner wall of the capillary, and the dipole orientation is an average of all orientations tangential to the capillary wall (usually, adsorbed fluorophores orient their absorption/emission dipole axis mostly paral-

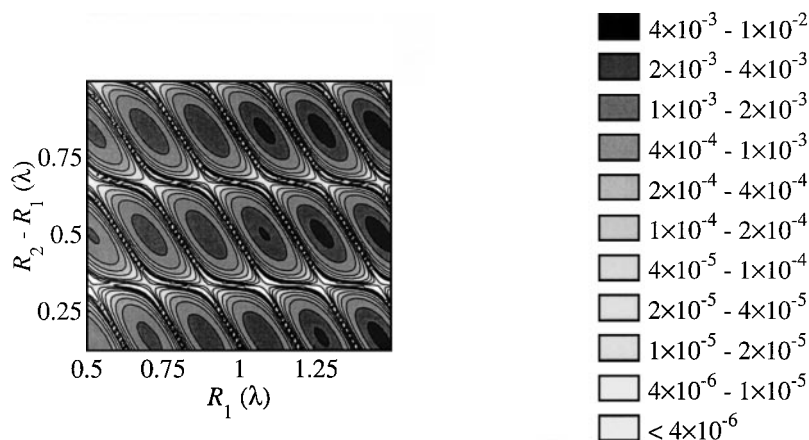


FIG. 2. Contour plot of the backscattering intensity for a laser beam polarized transversally to the axis of the capillary (x direction) in dependence of the values of the capillary radii R_1 and R_2 . The radii are given in units of the vacuum wavelength λ . The scattering intensities are normalized by the laser beam intensity along the y direction. Attention should be given to the logarithmic scale of the plot.

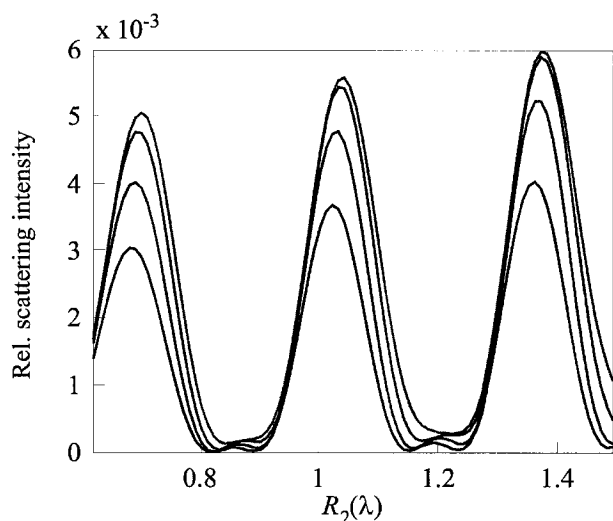


FIG. 3. Detected scattering intensities for different numerical apertures of the objective and different values of the outer capillary radius, R_2 , in units of the wavelength. From the lowest curve through the highest, the numerical aperture changes from 0.6 through 1.2 in steps of 0.2. All curves are normalized by the total laser beam intensity. The laser beam is transversally polarized (x direction).

lel to the adsorbing surface). In Figure 7, a plot for both cases for different values of R_2 is presented. If the diffusion of the fluorophores is not uniform over the whole cross section of the capillary but depends on the distance of the molecules from the glass surface (21), a weighted (rather than uniform) average has to be used for calculating the resulting lifetime changes, but the final result will be of the same order of magnitude as shown in Figure 7.

As can be seen, the lifetime change, if averaged over all dipole positions and orientations, is rather small. However, under favorable experimental conditions, it should be feasible to measure the change of the fluorescence lifetime for adsorbed dye molecules. An experimental approach that should readily be able to detect such small lifetime changes is the so-called burst integrated lifetime method (22,23). In this method, the fluorescence signal is

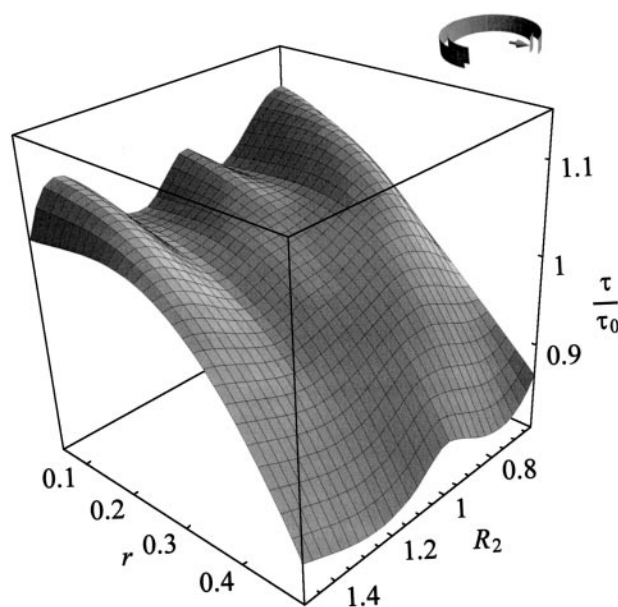


FIG. 4. Relative fluorescence lifetime of a dipole oriented along a radial axis for different distances r of the dipole from the axis of the capillary and for different outer capillary radii R_2 (both given in units of the emission wavelength λ in vacuum). The inner capillary radius is set equal to $\lambda/2$. The lifetime is normalized by the bulk lifetime in water.

collected and processed only for detected single-molecule fluorescence bursts, thus discriminating any signal that is collected outside a real single-molecule event. This allows one to discriminate huge background signals effectively (24). By accumulating the fluorescence of a sufficiently large number of single-molecule events, it should be possible to measure their lifetime with arbitrary precision.

To study the influence of the presence of the capillary on the fluorescence emission spectrum, calculations for a fixed capillary geometry but different emission wavelengths were performed. A typical result is shown in Figure 8. As can be seen, the change in lifetime remains rather small over the whole range of wavelengths. However, an interesting principal effect can be seen for the

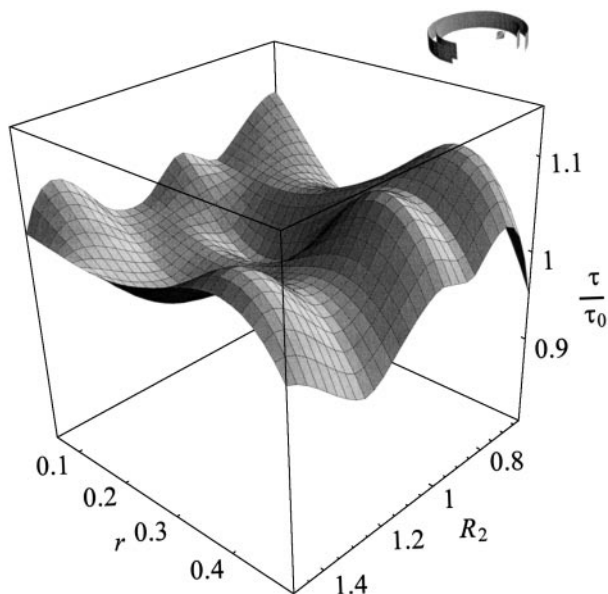


FIG. 5. Same as that shown in Figure 4, except that the dipole orientation is perpendicular to the axis and parallel to the circumference of the capillary.

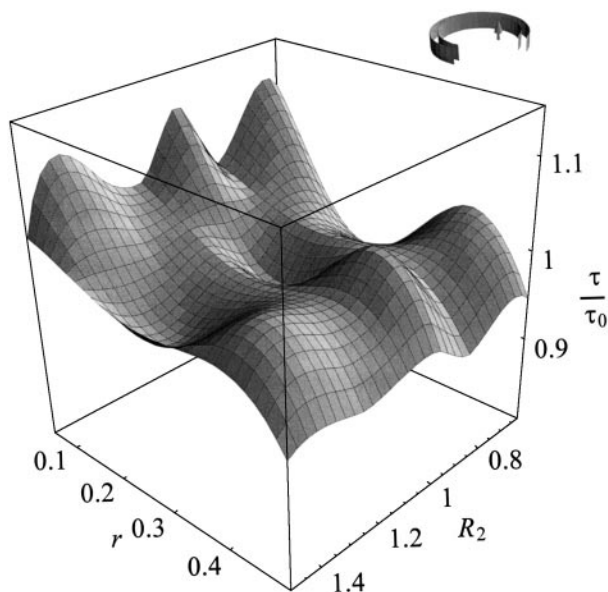


FIG. 6. Same as that shown in Figure 5, except that the dipole orientation is along the axis of the capillary.

lifetime change of molecules oriented along the y axis. With increasing wavelength, the lifetime change reverses sign: for smaller wavelengths, the lifetime is larger than the bulk lifetime; for larger wavelengths, it becomes smaller than the bulk lifetime. Thus, two molecules with different emission wavelengths can principally experience lifetime shifts into different directions. Such an effect may be of interest for applications in which one wishes to increase the difference in lifetimes between different molecular species (25,26). However, in such a case, one should

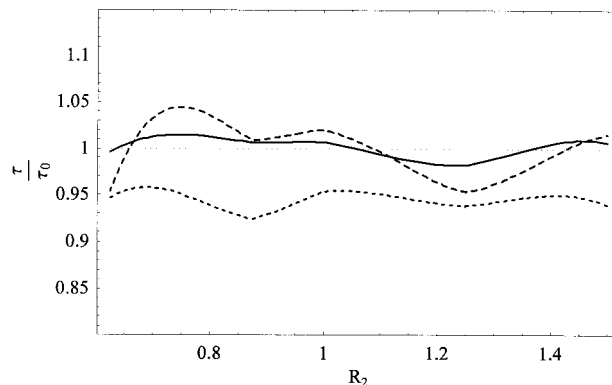


FIG. 7. Measurable fluorescence lifetime for freely diffusing dye (solid line) and for dye adsorbed to the capillary wall, with the dipole orientation perpendicular to (dashed line with long dashes) and along the (dashed line with short dashes) axis of the capillary. For comparison, the straight dotted line indicates the bulk lifetime in water.

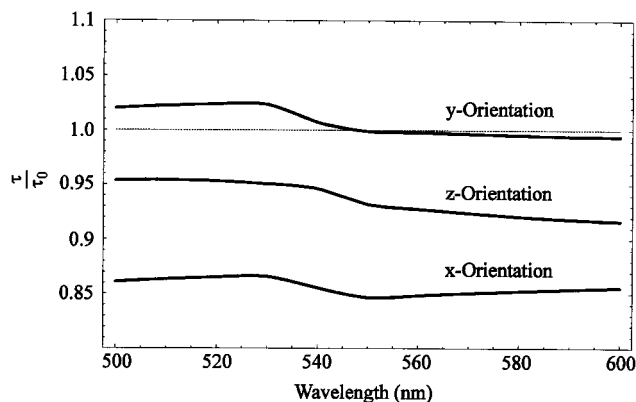


FIG. 8. Changes of the fluorescence lifetime of a molecule for different emission dipole orientations. The orientations refer to the coordinate system shown in Figure 1 and for a molecule on the x axes adsorbed at the inner capillary wall. The inner and outer radii of the capillary were set to $R_1 = \lambda/2$ and R_2 .

certainly consider using cavity configurations with much stronger electromagnetic coupling to the emission of the fluorophores.

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