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Tracking of fluorescent molecules diffusing within membranes

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Abstract. A new method is proposed for tracking fluorescing single molecules diffusing within a two-dimensional membrane. It is based on a confocal microscopy setup with a constantly rotating laser focus, which follows the position of the molecule. The optimization and efficiency of the method are theoretically studied for a broad range of experimentally realistic conditions. The proposed method allows for a long-time observation of diffusing molecules while allowing the application of fast spectroscopic techniques such as fluorescence decay time determination or fluorescence anisotropy measurements.

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In recent years, the fluorescence detection of single molecules (SMD) under ambient conditions within a liquid environment has seen a tremendous development, see for example [1–4]. This is partially due to the fact that versatile laser systems, ultrasensitive photoelectric detectors, and highly efficient optical components such as high-aperture microscope objectives have become widely available. Further, the ability to detect the fluorescence of individual molecules has triggered new research programs that were unthinkable without the ability to monitor and study molecules on a single-molecule level.

There are several techniques for detecting single-molecule fluorescence. Two of the most widely used techniques are confocal microscopy, and wide-field microscopy. An experimental technique similar to standard confocal microscopy was the first method successfully used for SMD, see [5] and citations therein. Due to the very small detection volume achieved using this method, the detected intensity of Rayleigh- and Raman-scattered excitation light can be kept low compared with the fluorescence signal generated by a single molecule, allowing for very high signal-to-background ratios. The disadvantage of this method, when detecting diffusing molecules, is that due to the small detection volume the average transition time of a molecule diffusing through the detection volume is very short (about 100 μs to several ms), limiting the time during which an individual molecule can be observed and studied. The most successful technique for studying the diffusion of fluorescent molecules is wide-field microscopy, where fluorescence is detected over a large spatial area (about $100 \times 100\,\mu m$), see for example [6–10]. The spatial resolution of this method in the sense of determining the molecule's position is far below the diffraction-limited resolution of the microscope, down to a few nm. However, the time resolution of conventional high-speed single-photon sensitive camera systems is limited to a few ms. Thus, they do not allow for studying fast molecular dynamics such as fluorescence decay or polarization anisotropy on a ns through μs time scale, which are very important when studying the interaction of a molecule with its environment, see for example [11–13]. For example, being able to detect the rotation of a fluorescent molecule by measuring fluorescence anisotropy changes with nano- and microsecond time resolution could reveal information about the local viscosity of a cell membrane [14]. Also, any change in the fluorescence decay time, which is usually in the ns time range, provides information about the change of local membrane properties that are quenching the fluorescence [15]. The standard technique for measuring fast fluorescence dynamics of single molecules is time-correlated single-photon counting (TCSPC) [12, 16, 17]. In this technique, fluorescence is excited by a train of short laser pulses, and one measures the delay times between the detection of a fluorescence photon and the exciting laser pulse. This method allows for the study of the fluorescence decay down to a few ps. However, for applying this technique, one needs a photodetector capable of continuously detecting single-photons with ps time resolution, which is far beyond the capabilities of usual camera systems. The standard detectors used for TCSPC are photomultiplier tubes and single-photon avalanche diodes, which do not provide any spatial resolution.

Thus, it would be advantageous to have a detection method for SMD that combines the high signal-to-background ratio and high temporal resolution offered by confocal microscopy with the ability of wide-field microscopy to observe and track individual molecules over a long time and distance. In the present paper, a new method is proposed and theoretically studied for tracking of single molecules moving within a plane, such as an artificial bilipid layer or a cell membrane.

It will be shown that the proposed tracking method can extend the detection time of single molecules by several orders of magnitude compared with a stationary confocal setup, without losing the ability to perform spectroscopic studies on an arbitrarily short time scale. To our knowledge, this is the first technique described in the literature that may allow such a long time spectroscopy of single molecules diffusing within a liquid phase. Thus, the proposed technique may be compared to the electromagnetic [18] or optical traps [19] widely used in high-vacuum single atom and molecule spectroscopy. Unfortunately, these traps are not applicable to single molecules having small dipole moments and diffusing within a liquid at room temperature.

1 Theory and numerical results

The general idea for the detection method to be proposed stems from a quite different field of research: the study of the motion of individual flagellate bacteria. In a seminal paper [20], Berg described an elegant approach for tracking the motion of single bacteria under a microscope. He observed free swimming bacteria with a camera attached to a microscope. However, instead of extracting the individual bacterium's motion from the recorded images by the help of some image-processing algorithm, the microscope stage bearing the microbial sample was moved in regular time intervals in such a way that the observed bacterium remained always within the center of the field of view. It was then possible to reconstruct the bacterium's motion by evaluating the microscope stage adjustments made.

The SMD method proposed here works in a similar way. Its general scheme is depicted in Fig. 1. The fluorescing molecules to be studied are considered to be confined within a plane, within which they can freely diffuse (for example fluorescently labeled proteins within a phospholipid cell membrane). A laser beam is tightly focused into this plane, and this laser focus is rotated around a circle. The numbers of detected photons are recorded during each rotation with sufficiently high temporal resolution (time binning being at least one order of magnitude smaller than the period of rotation).

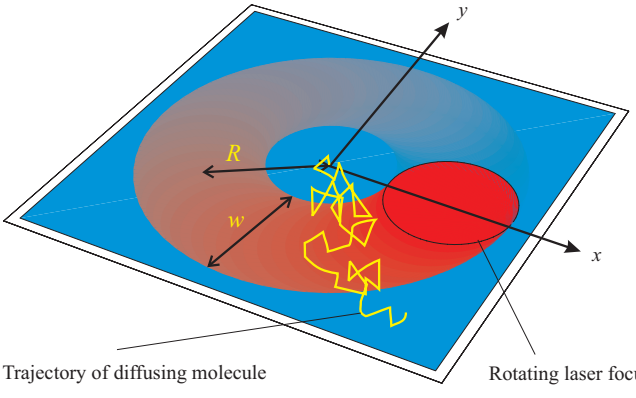

Fig. 1. General scheme of the proposed experimental setup. A Gaussian laser beam is tightly focused into the plane of diffusion of the molecule to be observed. The laser focus is moved around a circle with radius R. After each rotation, the center of the circle of rotation is adjusted to a new position according to the photon detection intensities observed along the rotation

After every rotation, the center of the circle is moved to new coordinates (x_c, y_c) according to the rules

$$x_c \mapsto \alpha \frac{N(x > x_c) - N(x < x_c)}{N_{tot}},$$
$$y_c \mapsto \alpha \frac{N(y > y_c) - N(y < y_c)}{N_{tot}}, \quad (1)$$

where x_c and y_c are the Cartesian coordinates of the circle's center within the plane, $N(x > x_c)$ is the number of detected photons along the focus' motion on the half circle with $x > x_c$ etc., N_{tot} is the total number of detected photons during one cycle of rotation, and α is an appropriate step width. By moving the center of the circle of rotation according to the rules of (1), the circle will follow the motion of any sufficiently slowly moving fluorescing particle, once the particle has first come close enough to the circle to give rise to a detectable fluorescence signal. There are three geometrical and one temporal parameters of this detection scheme: the diameter w of the laser focus that is assumed to have a Gaussian intensity profile, the radius R of the circle of rotation, the step parameter α, and the period T of one rotation.

The first question to be considered here is what are the optimum values of the geometrical parameters for tracking. In SMD experiments, one usually tries to keep the laser focus as small as possible to minimize the detection volume and thus the background scattering signal. In all subsequent calculations, the value of the focus diameter was always assumed to be 1 μm (close to diffraction-limited focusing for lasers at visible wavelengths). Furthermore, the larger the radius R of the circle is the better the spatial distribution of fluorescence is probed by the rotating laser focus. However, if that radius is larger than a critical value, a fixed point source at the center of the circle is less excited, when averaged over time, than another fixed point source some distance away from that center. If the tracking works well the tracked particle will be always close to the center of rotation. Thus, an optimum radius value will be as large as possible for optimally probing the spatial position of the particle, but without "under-exposing" the center of the circle. Consider the average light intensity $I(r)$ that a molecule, placed at a fixed distance r from the center, receives from the rotating laser focus. Let the spatio-temporal intensity distribution $P(x, y)$ of the moving laser focus be proportional to

$$P(x, y, t) \propto \exp\left(-\frac{2(x - R\cos(\omega t))^2 + 2(y - R\sin(\omega t))^2}{w^2}\right), \quad (2)$$

where $\omega = 2\pi/T$ is the angular frequency of the rotation, and (x, y) are Cartesian coordinates. Then $I(r)$ can be computed as

$$I(r) \propto \int_0^T \mathrm{d}t P(x, y, t) = T \exp\left[-\frac{2}{w^2}(r^2 + R^2)\right] J_0\left(\frac{4\mathrm{i}Rr}{w^2}\right), \quad (3)$$

where the relation $r^2 = x^2 + y^2$ holds, and J_0 denotes the zeroth-order Bessel function of the first kind. The shape of $I(r)$ for different values of R is shown in Fig. 2. For values of R slightly larger than $0.6w$ a local minimum of $I(r)$ occurs at

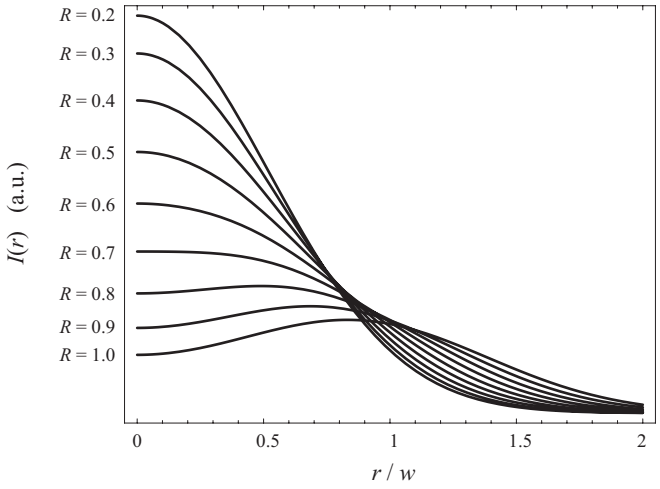

Fig. 2. Shape of the average intensity $I(r)$ received by a molecule at a fixed distance r (in units of w) from the center of rotation during one period of length T. Different curves correspond to different values of the rotation circle radius R (indicated on the left side of each curve)

$r = 0$. Thus, in all subsequent calculations, the value of R was chosen to be $R = 0.6w$.

The value of the step parameter α will be optimum if the adjustment steps as prescribed by (1) move the center of the circle as close to the actual position of the molecule as possible. Consider the special case of $x_c = y_c = 0$ and a molecule on the x-axis at a distance x_m from the center of the rotation circle. For small values of x_m, (1) shows that a fair estimate of the optimum α-value is given by

$$\frac{1}{\alpha} = \frac{\mathrm{d}}{\mathrm{d}x_m} \left\langle \frac{N(x>0) - N(x<0)}{N_{tot}} \right\rangle \Bigg|_{x_{m=0}}, \tag{4}$$

where the brackets denote temporal averaging. Notice that the N-values in (4) implicitly depend on the coordinate x_m of the molecule. When approximating the average of the fraction in (4) by the fraction of the averages, and assuming that the average number of detected photons is proportional to the excitation intensity as given by (2), an analytical result can be found for α which reads

$$\alpha = \frac{\pi w^2}{8R}. \tag{5}$$

Next, the efficiency of the tracking method for different values of T will be studied. The measure of the tracking efficiency can be defined as the time of how long it is possible to follow a molecule before 'losing' it (neglecting photobleaching here). In a real experiment, it has to be taken into account that not only photons caused by the molecule's fluorescence are detected but also background photons stemming from light scattering and dark counts of the detector and the electronics. Thus, by 'losing' the molecule it shall be understood the case when the detected number of photons during one period of rotation T is equal to or lower than the mean number of background photons $\overline{n}_{bg}$ detected during time T. For quantifying the tracking efficiency, Monte Carlo simulations of the proposed detection and tracking method were performed. In all calculations, the photon detection was always assumed to obey Poissonian statistics. One calculation always started with the molecule positioned at the center of the circle of rotation, and was stopped as soon as the number of detected photons during one period of rotation dropped below $\overline{n}_{bg}$. Thus, the final result of one calculation is the number of rotation cycles until the molecule is lost, which will be denoted by L. Repeating the calculation many times, one obtains the mean value $\overline{L}$ of this number for a given set of parameter values. As already mentioned above, the value of w was set to 1 μm, and the values of R and α were set both to 0.6 μm. The diffusion constant of the molecule was assumed to be $D = 10^{-2}$ μm^2/ms, a typical value for protein diffusion in cell membranes [21]. It will be shown below that the final numerical results can be related to other values of D by appropriate rescaling of the time scale. The average number of background photons detected during one period of rotation was always set to $\overline{n}_{bg} = 10$, a typical value for confocal SMD. Thus, there remained two free parameters: The period of rotation, T, and the fluorescence detection intensity, N_0. Values of T between $T = 1$ ms and $T = 20$ ms were studied. Finally, the values of the fluorescence detection intensity N_0 were chosen within a range of values so that $N_0 T$ varied between 50 and 500, corresponding to signal-to-background ratios between 5 and 50, also typical values for confocal SMD. It emerged that the T-dependence of $\overline{L}$ follows a $\exp(\beta/T)$-law, where β is a characteristic factor independent of T. This can be understood if one realizes that the probability that a molecule diffuses a fixed distance a away from some original position during time T is given by

$$\int_a^\infty \mathrm{d}r \frac{r}{4\pi DT} \exp\left(-\frac{r^2}{4DT}\right) = \frac{1}{2\pi} \exp\left(-\frac{a^2}{4DT}\right). \tag{6}$$

It can be expected that this probability is proportional to the probability of losing the molecule during one period of rotation, so that $\overline{L}$ should be proportional to the inverse of this probability. In Fig. 3, the determined dependency of $\overline{L}$ upon T is plotted for different values of $N_0 T/\overline{n}_{bg}$, and Fig. 4

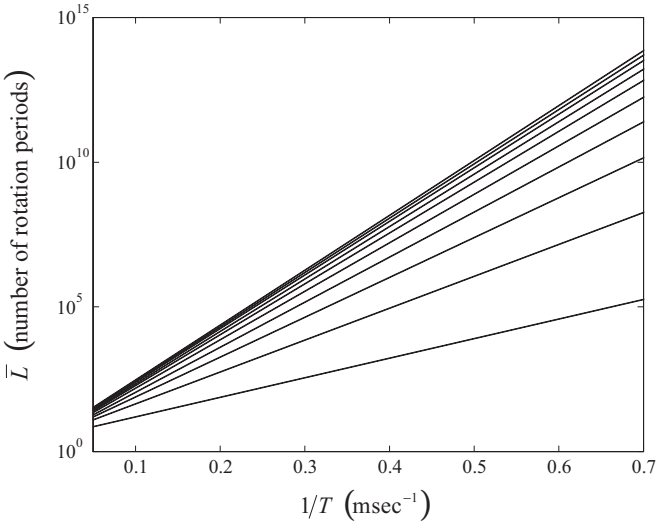

Fig. 3. Dependence of the mean number $\overline{L}$ upon the inverse of the period of rotation T^{-1}. Shown are curves for different signal-to-background ratios $N_0 T/\overline{n}_{bg}$. From the *bottom curve* to the *top curve*, the value of $N_0 T/\overline{n}_{bg}$ changes from 5 to 50 in steps of 5. The absolute value of $\overline{n}_{bg}$ was equal to 10 photons

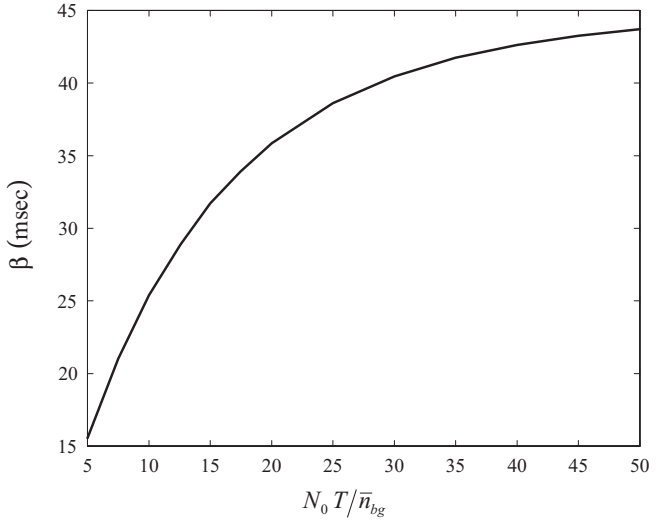

Fig. 4. Dependence of the characteristic exponential factor β upon signal-to-background ratio $N_0T/\overline{n}_{\rm bg}$ for $\overline{n}_{\rm bg} = 10$ and $w = 1\,\mu$m

shows the dependence of the characteristic factor β upon the signal-to-background ratio $N_0T/\overline{n}_{\rm bg}$. When interpreting these figures, one has to keep in mind that for the chosen simulation parameters, the characteristic time τ is 12.5 ms, being a rough estimate of the mean residence time of a diffusing molecule within a stationary laser focus with a diameter of 1 μm. The ratio τ/T is a measure of $\overline{L}$ for SMD without tracking, to which the $\overline{L}$-values determined for tracking have to be compared. Due to the $\exp(\beta/T)$-dependence, the values of $\overline{L}$ increase rapidly with decreasing time T.

The calculated numerical results can be readily adopted to other values of the diffusion constant. If the new value is denoted by D', then the results of Figs. 3 and 4 will not change when rescaling the time scale by a factor of D/D'. Of course, one has to take into account that by this rescaling, the detected photon intensities will also change if the exciting laser power is held constant. This will not affect the signal-to-background ratio, but the absolute values of N_0T and $\overline{n}_{\rm bg}$. However, by changing the exciting laser power by the same factor D/D', the two situations become completely equivalent. Thus, the found relations as depicted in Figs. 3 and 4 represent very general results for the tracking method.

For the worst studied signal-to-background ratio value of 5 and the diffusion constant $D = 10^{-2}\ \mu\text{m}^2/\text{ms}$, a rotation period of 1.4 ms corresponds to a mean tracking time value of 10^5 rotation periods or 140 s, compared to about 12.5 ms without tracking. Thus, the time one is able to follow a molecule is more than four orders of magnitude longer than without tracking. This situation improves with increasing signal-to-background ratio, and reaches its optimum for ratio values near 50, as can be seen from Fig. 4. These results were obtained on the assumption that the fluorescence emission has a strictly Poissonian character and that no photobleaching occurs. For many real fluorophores, effects such as blinking (repeated transition into non-fluorescent intermittent states) may occur, which will decrease the tracking efficiency. But even in that case tracking still promises an extension of the observation time far beyond its value for a non-tracked molecule. The ultimate limit of the tracking is, of course, always set by the photobleaching of the tracked molecule.

2 Conclusion

A new method of tracking a fluorescing single molecule diffusing within a two-dimensional membrane was proposed and theoretically studied. The proposed method offers the possibility of performing TCSPC measurements with high temporal resolution while following the molecule's motion. Thus, it offers a completely new direction of gaining information about molecule–membrane interactions or local membrane properties by measuring for example fluorescence anisotropy changes and/or excited state lifetimes.

The circular steering of the laser beam can be realized by existing techniques such as acousto-optical modulators or galvano-optical mirrors as used in confocal laser scanning microscopes. The adjustment steps for re-centering the rotating laser beam can be done by the same deflection technique or by an additional high-resolution displacement table, moving the whole sample with respect to the optics. The speed of the electronics necessary for fast photon counting and the adjustment calculations lies also within the proper range of existing counting cards and microprocessors. Thus, a technical realization of the proposed tracking method does not demand any non-standard technical components.

For initially finding a molecule it is sufficient to switch on the detection system and to wait until a molecule diffuses by chance into the detection region, similar to what one is doing in fluorescence-correlation spectroscopy. A more sophisticated initial scanning procedure for finding a molecule can also be envisioned. Once the detection system has encountered a single molecule, it will automatically track it until losing it, or until the molecule is photobleached. It should also be noticed that Fig. 3 yields information about upper limits of the tracking efficiency: if, for a given rotation period of the laser beam, molecules with given diffusion coefficient will be successfully tracked, on average, for some period of time, than any molecule with smaller diffusion coefficient will be tracked even longer. Thus, it is not necessary that the real molecule motion is a perfect Brownian diffusion (as assumed in the Monte Carlo calculation), one has to make only sure that the rotation period is small enough to keep up with the fastest motion of the molecules to be studied.

It would be interesting to extend the proposed method to the tracking of molecules diffusing in three dimensions. The limiting factor for doing so is not so much the ability to move a laser focus quick enough along a three-dimensional path, or the speed of the necessary calculations and adjustment steps. The main limitation will be the maximum number of fluorescence photons that can be detected within a given interval of time. This number is limited by the optical saturation of the detected fluorophore, leading to a maximum value of 10^3 detected fluorescence photons per ms under optimal conditions. Because tracking is performed on the basis of information gained by measuring the fluorescence intensity at different positions of the laser focus, tracking efficiency is directly correlated to the number of detectable fluorescence photons. It remains to be studied in future work whether a realistic tracking scheme can be devised allowing for single-molecule tracking in full three dimensions.

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