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LETTERS

Diffusion Measurements by Single-Molecule Spot-Size Analysis

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We present a method to obtain diffusion coefficients from single-molecule images in video microscopy experiments. This method is based on the size of the single-molecule spot and delivers even for short trajectories a maximum number of data points and a higher statistical accuracy than does the common mean square displacement analysis. It is thus possible to obtain a diffusion coefficient from a single snapshot and to follow slow changes in single-molecule mobility in diffusion constant trajectories. Random walk simulations of single-molecule spots are used to validate the method.

1. Introduction

During the last several years, single-molecule video microscopy has become an important tool to image and track single molecules in various environments.[1−4] Using this technique, one can detect single molecules with a positional accuracy of about 50 nm [5] and a temporal resolution in the millisecond range. Single-molecule video microscopy has been extensively used in bioscience to follow the translational mobility of membrane lipids,[1,6] to study myosin motion,[7,8] and to image conformational changes of DNA.[9] Extensions of this technique allow one to record the rotational motion of molecules[10] or to follow fluorescence resonant energy transfer.[11] Further applications in material science even demonstrate a 3D resolution obtained in combination with evanescent waves.[12] In many of these studies, diffusion constants determined from the trajectories are used to characterize the mobility of molecules. This is done by calculating the mean square displacement (MSD) as a function of time. Using the Einstein relation $\langle(\vec{r}(t) - \vec{r}_0)^2\rangle = 4Dt$ for two dimensions, the MSD can be connected with a diffusion coefficient D. Thus, one diffusion coefficient is commonly obtained from eq 1 for each trajectory, thus requiring a large number of molecules to be measured to obtain statistical significance.

$$D = \frac{1}{4}\lim_{t\to\infty}\frac{\mathrm{d}}{\mathrm{d}t}\langle(\vec{r}(t) - \vec{r}_0)^2\rangle \qquad (1)$$

Each trajectory in a single-molecule experiment is limited to a few snapshots mainly because of photobleaching. A typical number of snapshots in a trajectory ranges from 10 to 30 or, in exemplary cases, up to several hundred. Because the diffusion coefficient is defined as the limit of the MSD time slope (eq 1) when t approaches infinity, every trajectory of finite length is connected with an uncertainty in the determination of D. This has been extensively studied by Saxton[13] and Qian.[14] Because of these facts, a method that is capable of determining a diffusion coefficient for each trajectory is desirable. A possible ansatz, which we present here, is the spot-size analysis (SSA), where each snapshot of a single-molecule trajectory is analyzed to obtain a diffusion coefficient. This approach could increase ratio of the number of determined diffusion coefficients per trajectory to the number of snapshots in the trajectory and thus maximize the available data from the experiment.

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2. Description of the Method

Our method is based on the diffusional broadening of single-molecule spots in video microscopy experiments, which has been noted before by Schmidt et al.[3] and Xu et al.[4] without further analysis. Commonly, each molecule, which is spatially fixed during the image exposure time, acts as a point-like light emitter in a video microscopy experiment. Thus, molecules appear as diffraction-limited spots, and the spot size is given by the microscope's transfer function—the point spread function (PSF). However, if the molecule is moving during the exposure on a length scale that is comparable to the size of the PSF, its image will appear broadened. The spot size is then not given by the PSF but is given by a convolution of the PSF with an occupation frequency of certain positions during the exposure. This occupation frequency can be calculated from diffusion theory. If the diffusion process is governed by normal diffusion ($\langle(\vec{r}(t) - \vec{r}_0)^2\rangle \propto t$), then the probability density $p(\vec{r}, t)$ for finding a particle at a time t at a distance $r = |\vec{r}(t) - \vec{r}_0|$ from its origin $\vec{r}_0$ is written as

$$p(\vec{r}, t) = \frac{1}{8\pi Dt} \exp\left(\frac{-(\vec{r} - \vec{r}_0)^2}{4Dt}\right) \tag{2}$$

for two dimensions, assuming that $|\vec{r} - \vec{r}_0|$ is much larger than the mean free path length of the molecule. The conditional probability density that a position is occupied during the exposure time T is then given by the time integral of eq 2:

$$p(\vec{r}\,|T) = \int_{t=0}^{t=T} \frac{1}{8\pi Dt} \exp\left(-\frac{(\vec{r} - \vec{r}_0)^2}{4Dt}\right) \mathrm{d}t \tag{3}$$

By measuring the width

$$\sigma^2_{\mathrm{diff}} = \frac{\int_{-\infty}^{\infty} (\vec{r} - \vec{r}_0)^2\, p(\vec{r}\,|T)\, \mathrm{d}\vec{r}}{\int_{-\infty}^{\infty} p(\vec{r}\,|T)\, \mathrm{d}\vec{r}} \tag{4}$$

of eq 3 for different values of the diffusion constant D and the exposure time T, one finds the following relation for the case of 2D diffusion:

$$\sigma^2_{\mathrm{diff}} = DT \tag{5}$$

Thus, the size of the spot is proportional to the diffusion constant and the exposure time, as expected, because σ^2_{diff} measures a characteristic area of diffusion as the mean square displacement does.

Under experimental conditions, the distribution (eq 3) is convoluted with the *PSF* of the microscope.

$$I(\vec{r}\,|T) = p(\vec{r}\,|T) \otimes PSF \tag{6}$$

For simplicity, we approximate the *PSF* with a Gaussian function (eq 7).

$$PSF = \frac{1}{\sqrt{2\pi}\sigma_{\mathrm{G}}} \exp\left(-\frac{\vec{r}^{\,2}}{2\sigma^2_{\mathrm{G}}}\right) \tag{7}$$

We have carried out the convolution numerically for different diffusion coefficients and have obtained the relation between the broadened spot size σ_{Spot}, the size of $p(\vec{r}|T)$ σ_{diff}, and the size of the *PSF* σ_{G} using eq 8.

$$\sigma^2_{\mathrm{Spot}} = \sigma^2_{\mathrm{diff}} + \sigma^2_G \tag{8}$$

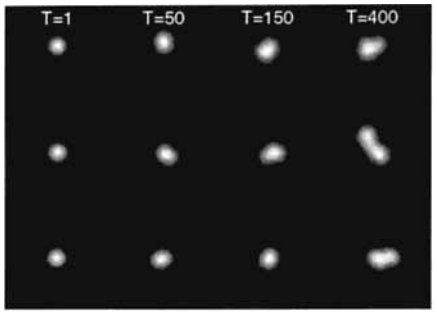

Figure 1. Simulated single-molecule spots from random walk simulations. Sample images for different exposure times T are depicted. Each column shows three different spots for the same exposure time. See the text for simulation details.

This equation will reproduce the *PSF* with the width σ_{G} in the limit of slow diffusion. So far, our considerations represent the time- or ensemble-averaged behavior with an infinite number of representations of the diffusional spot-broadening process. Each single-molecule exposure, however, is only a single realization of this process. The spot in a single-molecule exposure deviates from the ideal circular shape of the spots that are expected from the equations above and will instead be an image of a short trajectory smeared out by the *PSF*, which leads to two principal questions with respect to this method. First, how can the spot size be determined in a single-molecule experiment, and second, what is the uncertainty of this method?

To study these questions, we have employed random walk computer simulations of a diffusing particle. The random walk is carried out on a 2D lattice by allowing the random walker to take steps with a size of ± 1 or 0. The length of each trajectory is defined by TL_{T} where T is the number of steps during the exposure (the exposure time) and L_{T} is the number of exposures. During the exposure time, the occupation number of each lattice point is recorded. N_{T} realizations of each trajectory were carried out. The imaging process is modeled by convoluting the occupation number with a Gaussian function and projecting the result of this convolution on a lattice with a lower resolution that corresponds to the CCD array. The diffusion constant on the CCD array used throughout the simulations is $D = {}^1/_{48}$ pixel^2/step. The intensity of the image is defined to be proportional to the occupation number. Figure 1 shows several single-molecule images obtained for the same diffusion coefficient but for different exposure times T. It is clearly visible that for longer exposure times the spot deviates strongly from the circular shape.

To extract diffusion coefficients from such spots, one has to find an appropriate measure of the spot area as measured by σ^2_{spot} in the ensemble average. The measure has to be independent of the spot intensity and should show a simple linear relation to the diffusion coefficient and the exposure time of the form

$$A = A_0 + cDT \tag{9}$$

with A being the measured spot size, A_0, the diffraction-limited spot size, and c, a constant. The spots in Figure 1 suggest that an elliptical function would give an appropriate fit. We thus use a 2D Gaussian as a fitting function for each spot (eq 10).

$$I(\vec{r}) = I_0 \exp\left(-\frac{1}{2}\left[\frac{[(\vec{r} - \vec{r}_0) \times \vec{e}]^2}{\sigma_{\mathrm{s}}^2} + \frac{[(\vec{r} - \vec{r}_0) \cdot \vec{e}]^2}{\sigma_{\mathrm{l}}^2}\right]\right) \tag{10}$$

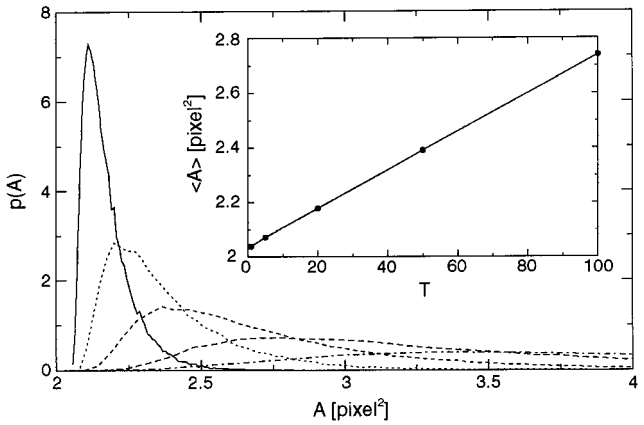

Figure 2. Distribution of spot sizes $A = \sigma_s\sigma_l$ obtained for the same diffusion coefficient $D = {}^1/_{48}$ pixel^2/time step but for different exposure times T (—, $T = 20$; ···, $T = 50$; - - -, $T = 100$; — — —, $T = 200$; · — · —, $T = 400$). The inset shows the dependence of the mean spot size $\langle A\rangle$ on the exposure time. The solid circles represent the calculated values. The line is a fit according to eq 11 with $A_0 = 2.04$ pixel^2 and $c = 0.33$.

Here, $\vec{e}$ is a unit vector along the long axis of the spot, and σ_s and σ_l are the widths of the Gaussian function along the short and the long axes, respectively. The area of the spot is then defined by the product $A = \sigma_s\sigma_l$. Fitting each exposure of a simulated trajectory for a given exposure time T results in a distribution of areas A as shown in Figure 2. The distributions are asymmetrically shaped and get broader for longer exposure times. However, the mean spot size $\langle A\rangle$ is a linear function of the exposure time, as displayed in the inset of Figure 2. Note that the linearity is a criterion for the method to give reasonable results. The linearity can be easily checked experimentally by varying the exposure time. Within the linear range the following relation between the diffusion constant and the exposure time applies:

$$D = \frac{A - A_0}{cT} \tag{11}$$

$A_0 = 2.04$ pixel^2 is the measured width of the *PSF* on the camera grid, and $c = {}^1/_3$. The distributions of areas shown in Figure 2 can thus be converted into distributions of diffusion coefficients. As required, these distributions are identical within errors, as depicted in Figure 3a.

So far, the distribution obtained from the SSA is rather broad, thus giving a large uncertainty in the measurement of D. However, the analysis now gives 1 diffusion coefficient per spot, which means that there are 50 diffusion coefficients for a trajectory with 50 snapshots compared to a single diffusion coefficient from the MSD analysis. Therefore, averaging over a number of diffusion coefficients from the SSA will narrow the distribution of diffusion coefficients if a single diffusion constant is present. As shown in Figure 3b, averaging over 5 spots will lead to the same statistical accuracy that was obtained for a trajectory of 50 snapshots from the MSD analysis. Averaging over the whole trajectory will give an even narrower distribution, with a total variation of about 25%. The distinction of a real distribution of diffusion coefficients from the uncertainties in their determination will thus be much easier with the SSA. This method is independent of the frame rate and allows the use of slow-scan CCD cameras.

To verify the theoretical results presented above, we have applied the method to experimental data. We have imaged the

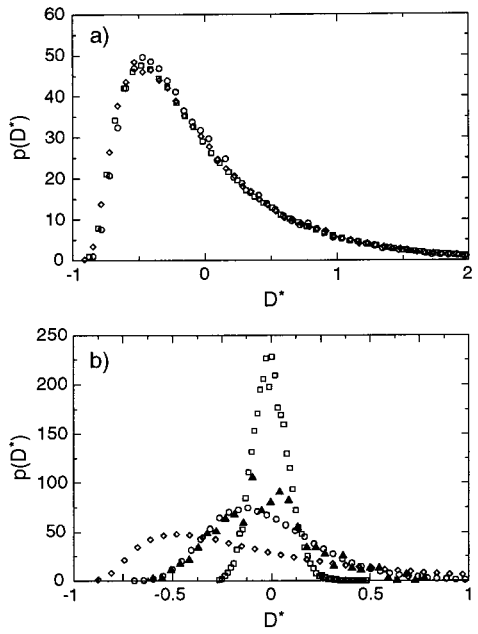

Figure 3. (a) Probability density of the relative diffusion constants $D^* = (D - \langle D\rangle)/\langle D\rangle$ obtained for different exposure times (○, $T = 20$; □, $T = 50$; ◇, $T = 100$). (b) Probability density of the relative diffusion constants $D^* = (D - \langle D\rangle)/\langle D\rangle$ obtained from the mean square displacement analysis (▲) and from the spot-size analysis with no averaging (◇) and averaging over 5 (○) and 50 spots (□) within a trajectory.

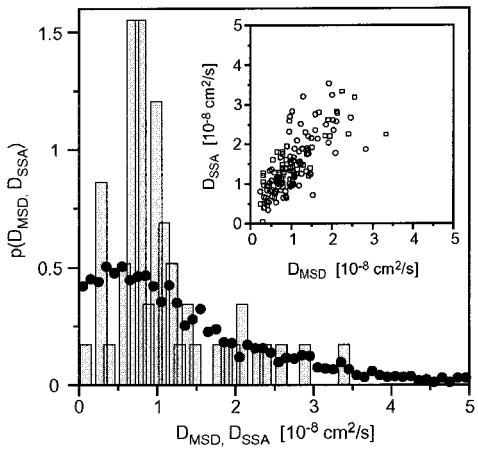

Figure 4. Probability density of diffusion coefficients obtained from single-molecule tracking experiments in a 4-nm tetrakis(2-ethylhexoxy)-silane film on a glass substrate (for experimental details, refer to the text). Bars represent the distribution obtained by mean square displacement analysis ($\langle D_{MSD}\rangle = (1.1 \pm 0.1) \times 10^{-8}$ cm^2/s). The distribution obtained from the same trajectories with the SSA is shown by filled circles and results in an average diffusion coefficient of $\langle D_{SSA}\rangle = (1.3 \pm 0.1) \times 10^{-8}$ cm^2/s. The inset shows the linear correlation between diffusion coefficients obtained by the MSD and SSA techniques for two different exposure times in the experiment (○, 25-ms exposure; □, 50-ms exposure).

motion of single rhodamine 6G dye molecules in an ultrathin liquid film (4 nm) of tetrakis(2-ethylhexoxy)silane on a glass cover slip with the help of a wide field-fluorescence microscope, as described in ref 1. Images were recorded every 25 or 50 ms (25 ms/50 ms exposure time). The trajectories of single molecules were reconstructed using homemade software. Both the mean square displacement and spot-size analysis were applied and yield the data shown in Figure 4. Both distributions of diffusion coefficients show good agreement with average diffusion coefficients of $\langle D_{MSD}\rangle = (1.1 \pm 0.1) \times 10^{-8}$ cm^2/s

and $\langle D_{SSA} \rangle = (1.3 \pm 0.1) \times 10^{-8} cm^2/s$. The inset of Figure 4 shows the correlation between both methods with a plot of D_{SSA} versus D_{MSD}. The data points for different exposure times show a linear dependence of D_{SSA} on D_{MSD} with a slope that is close to 1. The slight deviation of the slope from the ideal value is caused by the heterogeneity of the diffusion in the sample, which is the topic of a forthcoming paper.

3. Summary

In conclusion, we have presented a method that can be used to analyze the broadening of single-molecule spots in video microscopy images. With this method, it is possible to assign a diffusion constant to each snapshot of a single-molecule trajectory, thus increasing the amount of data considerably. The new method even allows one to follow directly the temporal changes in diffusion constants over a single-molecule trajectory. Averaging over a number of snapshots increases the accuracy of the diffusion measurement beyond the current accuracy obtained by the mean square displacement analysis with limited trajectory length. Because each snapshot is a diffusion measurement, the frame rate of the imaging CCD is irrelevant.

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