Three-dimensional orientation determination of the emission dipoles of single molecules: The shot-noise limit

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The power of three-dimensional orientation detection of single emitting dipoles using a sophisticated scheme with three detectors in a confocal microscope is quantitatively explored by means of Monte Carlo simulations. We show that several hundreds of photons are sufficient for a reliable orientation determination. In typical single-molecule experiments, time resolutions in the submillisecond range for orientation trajectories become accessible. Experimental data on fluorescent latex beads and single perylene monoimide molecules show that a properly aligned setup can perfectly reproduce the simulated data. The simulations and experimental data highlight the potential of our method and give practical guidelines for its application. © 2008 American Institute of Physics. [DOI: 10.1063/1.2971183]

I. INTRODUCTION

Fluorescence techniques have seen rapid development in life sciences over the past decades for the tracing of proteins, DNA, and lipids in the cell. A fluorescent tag is usually introduced, which can be followed by microscopy techniques such as epifluorescence microscopy or by laser scanning microscopy. This could be boiled down to the visualization of single fluorescent entities in living cells.1–3 However, a single molecule cannot only be used as a position-reporting tag. Due to the anisotropy of absorption/emission of the nanoscopic antenna a single dye molecule is representing, it may also report on the orientation of the feature it is attached to.4 The orientation determination of single molecules has therefore gained a lot of attention in the past. Several different methods have been suggested and demonstrated. In the simplest approach, polarization techniques are employed to determine the component of the absorption/emission dipole in the plane perpendicular to the optical axis.5–8 More sophisticated techniques are necessary to gain information on the angle between the dipole and the optical axis, usually referred to as three-dimensional (3D) orientation determination. These techniques can be grouped into two principally different classes: interference pattern techniques and intensity distribution techniques. In the first class, the observation of characteristic patterns of dipole excitation in scanning confocal optical microscopy (SCOM) or of dipole emission3,14 is explored. The orientation is obtained by fitting the measured patterns to model functions. In the second class of techniques, either the longitudinal component of the exciting laser field is altered and the corresponding change of the excitation rate analyzed5,16 or the emission detected by a high numerical aperture (NA) microscope objective is distributed onto several detectors in such a way that the longitudinal component of the dipole emission can be determined.17 Most of the techniques suffer from a low time resolution, preventing their application for the observation of fast rotational processes. Inspired by a technique proposed by Fourkas,18 we recently demonstrated a technique with a time resolution that is shot-noise limited, i.e., the only limiting factor is the number of photons detected per time unit.19

Here we give a quantitative assessment of the shot-noise limit, i.e., we investigate how accurate a given dipole orientation can be reproduced and how this accuracy depends on the actual orientation, the number of detected photons per time unit, and the background level. The number of photons can be directly related to the achievable time resolution.

To this end, Monte Carlo (MC) simulations are performed. The advantage of MC simulations is that the emission dipole can be arbitrarily set, which is very difficult to realize experimentally. However, the agreement between simulations and experimental conditions needs to be tested. For this purpose, fluorescent latex beads are imaged because of their well-defined emission characteristics. Using single molecules as a test system is hampered by lack of an independent method to determine molecular orientation, i.e., a method not based on fluorescence emission/absorption. We show, however, that fluorescence based single-molecule dipole orientation determination is possible with a low number of photons.

II. MODELS AND METHODS

The angular dependent emission intensity $I_E$ of a transition dipole in a homogeneous medium in spherical coordinates can be written as

$$I_E = (\sin \theta \cos \phi e_x + \sin \theta \sin \phi e_y + \cos \phi e_z)^2,$$  

(1)

with $\theta$ the polar angle between the dipole axis and the $z$ axis.
and \( \phi \) the azimuthal angle. The dipole emission intensity can be interpreted as a probability distribution of detecting emitted photons in a certain direction. If, for example, the orientation of the emission dipole coincides with the z axis, most of the photons will be emitted close to the x, y plane, and the probability of detecting a photon along the z axis is essentially zero. The main idea of the 3D orientation determination of the emission dipole is to make use of the anisotropic nature of dipole emission. Therefore, the ratio of the integrated detection probabilities in two distinct angular regions or, in terms of numerical aperture, in two NA regions is determined. These regions are defined by the detection scheme depicted in Fig. 1(a). The fluorescence light collected by the microscope objective is split by an annular mirror and a polarizing beam splitter into three components. The annular beam splitter is a mirror with an elliptical aperture and reflects the high-angle emission, whereas low-angle emission passes through the aperture. Low-angle emission is further split into two orthogonally polarized contributions. All emission components are focused onto avalanche photodiodes.

Figure 1(b) shows a more detailed view of the relation between the dipole orientation and the fractions of light directed to the detectors. The low-angle region is defined by \( 0 < \theta < \alpha_c \), where \( \alpha_c \) is given by the radius of the aperture of the annular beam splitter. The high-angle region is defined by \( \alpha_c < \theta < \alpha_r \), where the rim angle \( \alpha_r \) is given by the opening angle of the microscope objective. Here, by using a water-immersion objective with \( \text{NA}=1.2 \), this angle results in \( \alpha_r = 64^\circ \). For the determination of \( \theta \), two limiting cases can be discussed according to Figs. 1(c) and 1(d). For \( \theta = 0^\circ \), the emission in the low-angle region reaches its minimum, whereas for \( \theta = 90^\circ \) the fluorescence emission in the low-angle region is maximized. The fraction of light emitted into the high-angle region remains almost constant for both cases.

The emission intensities in the low-angle (central) region \( I_c \) and in the high-angle (rim) region \( I_r \) can be expressed according to 18,19

\[
I_r(\theta, \phi) = 2I_{tot}(t, t + \tau)[(A_r - A_c) + (B_r - B_c)\sin^2 \theta], \tag{2}
\]

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A. Simulations of three-dimensional orientation determination

Equations (2)–(4) can be interpreted as the probabilities of detecting a photon in the respective detector as a function of the orientation of the emission dipole if the total emitted intensity is normalized to $I_{0}=1$. In order to account for the shot-noise nature of dipole emission, single-photon events are generated by MC methods in the following way. The range between 0 and 1 is divided into three intervals with widths corresponding to the respective detection probabilities and one additional interval accounting for no photon detection. Random numbers $r$ between 0 and 1 are drawn and photons assigned to the detector where the random number $r$ falls in the respective interval. In this way, the averaged intensity ratios according to Eqs. (2)–(4) are maintained for a given input orientation. Background photons are simulated accordingly by assuming an isotropic angular distribution, i.e., the respective probabilities are just the steradians normalized by $4\pi$. This approach of simulating single-photon events resembles the experiment to the highest extent and assures maximum flexibility for subsequent analysis. Therefore, a constant number of photons was simulated to obtain an orientation dataset. Such an orientation dataset can be assigned to the number of photons detected within the integration time in an experimental setup.

To analyze the simulated data, the three photon numbers of each bin are fed as intensity triplets into Eqs. (9) and (10), yielding apparent orientations characterized by pairs of azimuthal and polar angles $\theta$ and $\phi$. For input orientations with azimuthal and polar angles close to 0° and 90°, the arguments of the square root function or of the inverted trigonometric functions in Eqs. (9) and (10) accidentally take values outside the respective domains due to shot noise. Those values can be either discarded from the analysis or attributed to the closest allowed argument, i.e., ±1 or 0, respectively. The latter procedure, which we call healing, results in an apparent orientation for the invalid dataset characterized by polar or azimuthal angles of 0° or 90°, respectively, i.e., the limiting cases discussed above.

For a statistical analysis of the apparent orientations, 200 datasets are created for each input orientation. The apparent orientations can then be compared to the input orientations of the experiment. All simulations were carried out using IGOR PRO (Wavemetrics).

B. Orientation resolved confocal microscopy

The experiments were performed on a homebuilt sample SCOM (Ref. 19) equipped either with a water-immersion microscope objective with NA=1.2 or with an oil-immersion objective with NA=1.4. A homemade mirror with an elliptical aperture was used to separate the angular regions of emission. The diameter of the projected circular aperture was 3 mm, corresponding to cutoff angles of $\alpha_{c}^{\text{oil}}=45^\circ$ and $\alpha_{c}^{\text{water}}=55^\circ$ in the case of the water- and oil-immersion objectives, respectively. A polarizing beam splitter (Melles Griot) was used to split the low-angle emission into its components. Samples were prepared by spin casting either aqueous solutions of fluorescent latex beads (diameter of 100 nm, Sigma-

$$I_{0,c90',c}(\theta, \phi) = I_{0}(t, t + \tau) \left[ A_{c} + B_{c} \sin^{2} \theta \right.$$ 0°, $c$ $$\pm C_{c} \sin \theta \cos 2\phi \right]_{90', c},$$ (3)

$$I_{e}(\theta, \phi) = I_{0,c}(\theta, \phi) + I_{90',c}(\theta, \phi)$$ $$= 2I_{0}(t, t + \tau)[A_{c} + B_{c} \sin^{2} \theta],$$ (4)

with $I_{0}$ as the total emitted intensity integrated over all directions in space for a given time period and the following variables:

$$A_{c,r} = \frac{1}{6} - \frac{1}{3} \cos \alpha_{c,r} + \frac{1}{12} \cos^{3} \alpha_{c,r},$$ (5)

$$B_{c,r} = \frac{1}{8} \cos \alpha_{c,r} - \frac{1}{8} \cos^{3} \alpha_{c,r},$$ (6)

$$C_{c,r} = \frac{7}{48} - \frac{1}{16} \cos \alpha_{c,r} - \frac{1}{16} \cos^{2} \alpha_{c,r} - \frac{1}{48} \cos^{3} \alpha_{c,r},$$ (7)

which are fully defined by the detection angles $\alpha_{c}$ and $\alpha_{r}$. Therefore, the detectable intensity according to

$$I_{D} = I_{e} + I_{r} = 2I_{0}(t, t + \tau)[A_{c} + B_{c} \sin^{2} \theta]$$ (8)

is only a function of the polar angle $\theta$. With the common definition of the polarization $P$ as $P := (I_{0,c} - I_{90,c})/(I_{0,c} + I_{90,c})$ and defining corresponding an inclination $N$ as $N := (I_{e} - I_{r})/(I_{e} + I_{r})$, the polar angle $\theta$ and the azimuthal angle $\phi$ can be calculated according to

$$\theta = \arcsin \left( -\frac{2A_{c} - A_{r} + A_{N}}{2B_{c} - B_{r} + B_{N}} \right)$$ (9)

and

$$\phi = \frac{1}{2} \arccos \left( -P \frac{A_{c} + B_{c} \sin^{2} \theta}{C_{c} \sin^{2} \theta} \right).$$ (10)

$N$ and $P$ are invariant against the sign of $\theta$ and $\phi$. This leads, together with the symmetry of dipole emission, to an eightfold orientation degeneracy for a given $\{P,N\}$ pair and therefore to a range of $\theta$ and $\phi$ from 0° to 90°.

In order to find the optimal value for $\alpha_{c}$, we consider the range of possible values $N(\alpha_{c}, \alpha_{r}, \theta) = (I_{e} - I_{r})/(I_{e} + I_{r})$. Therefore, the contrast $\Delta N$,

$$\Delta N = \sqrt{(N_{\theta=0} - N_{\theta=90})^{2}},$$ (11)

is maximized with respect to $\alpha_{c}$ as shown in Fig. 1(e) with a fixed rim angle of $\alpha_{r}=64^\circ$. This results in an optimal cutoff angle of $\alpha_{c}^{c}=43^\circ$. For this cutoff angle, $N$ takes values between $N_{\text{min}}=0.09$ and $N_{\text{max}}=0.49$. With these numbers, the detectable intensities $I_{e} = (I_{0,c} + I_{90,c})$, $I_{r}$, and $I_{D}=I_{e} + I_{r}$ can be plotted as a function of $\theta$ as shown in Fig. 1(f). The change in $N$ as a function of $\theta$ is mainly governed by the increase in $I_{e}$. Note that the total detectable intensity $I_{D}$ as a function of $\theta$ varies between 15% and 25% of the light emitted by a single fluorophore for a NA=1.2 water-immersion microscope objective.
Aldrich) or cosolutions of perylene monoimide (PMI) (Sigma-Aldrich) and polymethylmetacrylate (GoodFellow) in toluene (Uvasol, Merck) onto glass cover slips. Prior to spin casting, the cover slips were heated for 1 h at 510 °C to remove fluorescent contaminants.

III. RESULTS OF SIMULATIONS

Figures 2(a) and 2(b) show apparent orientations versus input orientations for a simulation with 500 photons per dataset. The input orientation was varied from 0° to 90° in steps of 5° for all combinations of θ and φ. In Fig. 2(a) the apparent azimuthal angle θ is shown with the two possible ways of dealing with the intensity triplets that lead to ill-defined angles: discarding and healing. For a wide range of the input azimuthal angles (10° < θ < 80°), 500 detected photons per bin are already sufficient to reproduce the input orientation with high accuracy and a standard deviation of around 5°. For azimuthal input angles close to 0° and 90° the apparent angles significantly deviate from the expected values and the percentage of valid bins drops drastically. If these datasets are healed by assigning the respective limits of N and P, however, the input angles are significantly better reproduced by the analysis. It should be mentioned that the apparent azimuthal angle is independent of the polar angle due to rotational symmetry. Quite the opposite holds for the apparent polar angles shown in Fig. 2(b). Here, only analysis with healing invalid bins is shown. The reproducibility of φ depends strongly on θ: For dipoles oriented almost parallel to the optical axis (small θ), the apparent φ is 45° for any input value, and the standard deviations are huge. This behavior can be intuitively understood by taking into account that the polarization measures the projection of the dipole into the sample plane, which is vanishing for small θ. However, one should keep in mind that, in the case of a completely random distribution of dipole orientations, small angles of polarization should keep in mind that, in the case of a completely random distribution of dipole orientations, small angles of polarization.

As can be seen in Figs. 2(c) and 2(d), only 100 detected photons per dataset allow for reasonable determination of θ and φ for a wide range of azimuthal input angles with an error of 15°. This is a remarkable result, showing the potential for the obtainable time resolution for orientation determination with the proposed detection scheme. The deviations close to the borders of the respective intervals are slightly larger for θ as compared to φ. For 3200 detected photons per bin the error

FIG. 2. (Color online) Results of MC simulations of 3D orientation determination (see text for details). (a) Apparent polar angle vs input angle for two types of analysis: without (circles) and with (triangles) healing of invalid datasets. The percentage of invalid datasets is also plotted (squares). (b) Apparent azimuthal angle vs input angle with healing for different polar angles. The arrow indicates increasing polar angles (0°, 10°,...,90°), i.e., larger in-plane components of the emission dipole. A constant number of 500 photons per dataset was used throughout. [(c) and (d)] Influence of the number of photons per dataset on the reproducibility of the polar (c) and azimuthal (d) angles. The arrow indicates an exponential increase from 100 to 3200 photons per dataset. [(e) and (f)] Influence of background contribution on the reproducibility of the polar (e) and azimuthal (f) angles. The arrow indicates increasing background fraction from 0% to 100%.
has decreased to less than 5°, and input angles close to 0° and 90° can be reproduced with high accuracy. So far, no background photons were included.

The last point in this section addresses the influence of the isotropic background on the determination of a given orientation. The apparent azimuthal angles θ versus input angles with varying background contribution are shown in Figs. 2(c) and 2(f). A constant number of 500 photons per dataset was used throughout here. This number is composed of the intensity triplet from the anisotropic dipole emission and an additional intensity triplet of the isotropic background emission. The background level was varied from 0% to 100% in steps of 10%, representing the fraction of photons per bin attributed to background fluorescence. As shown in Figs. 2(e) and 2(f), a background level of 10%, which is rather large compared to signal-to-background ratios accessible with confocal microscopy, reproduces the input angles reasonably well. If the background level is further increased, the distribution of photons into the three detectors will be dominated by the background photons. In this case, the apparent angles approach the values for isotropic emission, which amount to θ=57° for the polar angle and φ=45° for the azimuthal angle. The standard deviation, which is mainly dependent on the number of photons per dataset, is constant with an increasing background level and cannot be used as a criterion to distinguish between a low or a high level of background. If the background contribution can be measured as a reference, it can be simply subtracted, eliminating the deviations caused by the background.

We would like to stress here that Fig. 2 can be used to correct the apparent angles for shot noise and to obtain the proper determination of the molecule’s orientation in an experiment.

From the photon numbers the potential time resolution of the determination of the orientation of single molecules can be estimated. Let us assume a detectable count rate of 200 kHz for an immobilized molecule with an arbitrary orientation of the emission dipole. Already with 100 photons per integration time, the 3D orientation can be determined with reasonable accuracy. For this count rate 100 photons per bin correspond to a time resolution of 500 μs.

IV. RESULTS OF EXPERIMENTS

In order to test the agreement of simulation and experiment, fluorescent latex beads were used, which were immobilized on the cover glass surface. We would like to stress here again that an experimental realization of the simulations presented in Fig. 2 is impeded by lack of a technique to control the orientation of single dye molecules. Therefore, a test system with at least well-defined emission characteristics is needed. Fluorescent latex beads as isotropic emitters are therefore well suited for experimental validation of the simulations. It should be mentioned here that the emission distribution of a dipole emitter close to an interface between media with different refractive indices is altered as compared to an isotropic surrounding. The interface between water and glass, where the beads are located, however, does not significantly influence the isotropic emission because the vast majority of emitters within the bead mostly feels the surface of the bead, which is a sphere. The concentration of the beads was chosen in such a way that after immobilization the mean interbead distance was large enough to clearly separate single beads with the confocal microscope.

Figures 3(a) and 3(b), show the per-pixel determined φ and θ values obtained by raster scanning a bead sample with our confocal detection scheme. To match the requirements of the water-immersion objective, the beads were covered with...
water. No desorption of latex beads was observed. The beads were excited with circularly polarized light with an intensity of 10 nW generated by a laser operating at 488 nm. Behind the dichroic mirror, a long pass filter with an edge at a wavelength of 500 nm was used to block the remaining laser light. An area of $5 \times 5$ $\mu m^2$ was raster scanned using the piezo-driven scanning stage with a resolution of $128 \times 128$ pixels with an integration time of 2 ms per pixel. Three intensity images were obtained (data not shown) for each scan representing $I_{y,x}$, $I_{90\degree,x}$, and $I_c$, which were used to calculate pixel by pixel the $\theta$ and $\phi$ values according to Eqs. (9) and (10). The orientation images of all beads are rather uniform for both angles, indicating the reliability of the method. Additionally, the obtained angles for all pixels of one bead were averaged. The obtained averages indicated in Figs. 3(a) and 3(b), for each bead are within a very small interval. The isotropic emission of the beads allows for a correction of $I_c$ by weighting of the photons detected in one of the two corresponding detection channels for ($I_{y,x}$ and $I_{90\degree,x}$), which can be changed in such a way that the histogram of $\phi$ values is centered at $\phi_{av}=45\degree$ (data not shown). This weighting factor amounts to 1.1 for the present experimental setup. For the correction of $\theta$, a different approach was used. The ratio of the low-angle and high-angle intensities ($I_l$ and $I_h$) is determined by the diameter of the elliptical aperture of the mirror. Therefore, instead of weighting the number of detected photons, the cutoff angle $\alpha_c$, which is used for the calculations, can be adjusted in such a way that $\theta_{av}=60\degree$ is obtained. This correction led to $\alpha_c=41\degree$, in perfect agreement with the theoretical value.

The remaining key question is whether also the shot noise in the experiment corresponds to the simulation. Therefore the transiently recorded photons from a bead centered in the laser focus were analyzed. Figures 3(c) and 3(d) show $\theta$ and $\phi$ histograms of datasets extracted from the detected photon stream. A bin time of 1 ms was chosen, corresponding to $\sim150$ photons per dataset. For comparison, histograms of accordingly simulated datasets are shown in Figs. 3(a) and 3(b). As input parameters for the MC simulations, $\alpha_c$ was set to $41\degree$ and each dataset was simulated using $I_{tot}=750$ photons. Taking into account that, according to Fig. 1(f), only about 20% of the overall number of emitted photons can be detected, this corresponds to the number of around 150 photons/ms as detected in the experimental case. No further background was added. The normalized histograms of the experimental data coincide almost perfectly with those obtained from MC simulations. We therefore conclude that the experimental realization of our method is in excellent agreement with theory. The results of the MC simulations for single emitters can thus be used as a reference for experimental data.

In order to test qualitatively whether our method allows for 3D orientation determination at the single-molecule level with a low number of photons, we investigated single PMI molecules embedded in a polymer film. Figures 4(a) and 4(b) show the $\theta$ and $\phi$ images obtained by raster scanning the sample and calculating pixelwise the respective angles. The molecules were excited with circularly polarized laser light with a power of 5 $\mu$W at 488 nm, and the integration time per pixel was 2 ms. An intensity threshold of 50 photons was applied before analysis of the images. The analysis was performed with healing of invalid bins. As expected for preferentially randomly oriented molecules, small polar angles are rare. The azimuthal angles are distributed over the whole interval. The well-separated spots feature a relatively uniform orientation of the pixels. If two molecules are close together, the photons of one molecule influence the observed orientation of the neighboring molecule. As for all orientation determination techniques, the molecules should therefore be well separated to obtain reliable orientation data. Although it is not possible to quantify the error in orientation determination at the single-molecule level due to lack of a priori knowledge of the actual orientation, we are able to test the experimental noise against the simulated shot noise. Therefore, in Fig. 4 the transient intensities and orientations of one molecule from the experiment (c) are compared with accordingly simulated data (d). The input angles for the simulation were found by looking up the average apparent angle obtained from the analysis in Fig. 2. The molecule with the transients shown in Fig. 4(c) is difficult in the sense that the actual value for the azimuthal angle $\phi$ is close to zero. This results in a considerable number of invalid bins, which result in the gaps visible in the angle transients. The striking similarity of both the transients and the histograms of the experimental and simulated angles demonstrate shot-noise limited performance of the microscope. The sudden drop in fluorescence in Fig. 4(c) indicates photobleaching of the molecule. From the intensities before/after bleaching, the signal-to-background ratio can be estimated to be roughly 10:1. Here, the excitation rate was reduced to prevent rapid photobleaching, thus allowing for sufficient statistics for the comparison with the simulated transients. However, if a higher time resolution is required, the excitation rate can be increased to collect the same number of photons in a shorter time interval.

V. DISCUSSION

In principle, our technique of combined emission distribution and polarization detection (EDPA) is a mixture of Fourkas’ approach and of the direct emission pattern imaging (DEPI) demonstrated by Lieb et al. because we exploit polarization information for the determination of $\phi$ as well as emission intensity distribution for the determination of $\theta$. No quantitative treatment of the number of photons needed for orientation determination nor for the respective accuracy is given for both methods. We can therefore only qualitatively or semiquantitatively compare our approach.

As for the DEPI technique, also in our case slight defocusing will not affect the determined orientation, which is a big advantage compared to defocused pattern imaging (DPI) as discussed by Lieb et al. Furthermore, EDPA is like DEPI, less susceptible to optical aberrations in the microscope objective. Another advantage of both methods is that molecules that are close together can still be investigated, whereas the overlapping patterns in DPI prevent its application. The
larger errors for orientations with the dipole axis almost perpendicular to the optical axis are common to both EDPA and DEPI.

However, the key advantage of EDPA over DEPI and DPI is that no imaging device is required, which has two consequences, a technical and a principal. Imaging requires a camera, which still has a time resolution in the millisecond range, far apart from what can be achieved with single-photon detectors. The principal consequence of imaging on many pixels is that the sparse photons of a single molecule are distributed in space and in time over those pixels. If a signal to noise ratio of 1:10 per pixel is needed, on average 100 photons per pixel are required. For a pattern of 13 × 13 pixels, therefore, roughly 10 000 photons need to be detected, limiting the obtainable time resolution essentially. In contrast, the EDPA approach allows the orientation determination with a few hundreds of photons per time unit giving access to orientation trajectories in the submillisecond time range.

Our EDPA methods shares its advantage of the use of only a few detectors with the polarization analysis (PA) method proposed by Fourkas. However, to our knowledge this method has not been experimentally tested so far, probably for practical reasons. It is not that simple to split the light in three polarization components as it is required for PA. Additionally, our detection scheme has a higher symmetry, reducing the potential for systematic errors in the orien-

FIG. 4. (Color) Results of experiments with single dye molecules (PMI) embedded in a poly(methylmetacrylate) film. ([a] and [b]) Color-coded images of (a) the polar and (b) the azimuthal angles. (c) Experimental and (d) accordingly simulated transient orientation of a single PMI molecule centered in the laser focus. The totally detected fluorescence intensity is also plotted showing single-step bleaching behavior for the PMI molecule. The integration time per bin was 5 ms. ([e] and [f]) Distributions of (e) polar and (f) azimuthal angles from datasets obtained from the experimental bars and simulated lines transients ([c] and [d], respectively).
tation determination. On the other hand, EDPA and PA also share one disadvantage, namely, the higher degree of degeneracy as compared to DPI and DEPI. If there is no a priori knowledge of possible dipole orientations, this degeneracy will definitely hamper the application of EDPA in terms of unambiguous orientation determination for stationary emission dipoles.

We would like to point out that the single-photon based orientation determination in EDPA and PA allows for investigating rotational diffusion via correlation functions down to the nanosecond timescale, which is impossible at all with DEPI or DPI.

VI. CONCLUSION

By means of MC simulations we have explored the limits of our previously demonstrated method for the determination of the spatial orientation of single dye molecules. We have shown that 500 detected photons are sufficient for the determination of the orientation with reasonable accuracy as long as the actual orientation is neither nearly parallel nor perpendicular to the optical axis. Therefore, in experiments attention should be paid to both angles. Depending on the desired accuracy and on the excitation rate, a high time resolution for orientation determination can be achieved. In conjunction with the simplicity of the approach without the need for time-consuming fitting procedures as it is the case for other methods proposed in literature, a real-time orientation determination becomes feasible, paving the way for sophisticated orientation manipulation techniques.

Experiments with fluorescent latex beads and single fluorescent molecules have shown that the simulated data can be reproduced with high accuracy if all optical components are properly aligned. Behind the shot-noise nature of dipole emission, the accuracy of the method for experimental applications/use is mainly determined by the annular mirror, which is used to distribute the fluorescent light. To improve the accuracy, a telecentric lens system could be used to broaden the beam behind the pupil of the objective. This would allow the use of an annular mirror with a larger opening and the placement of the mirror in an image plane of the aperture of the microscope objective, further improving the accuracy of the method.

In summary, the proposed detection scheme is a robust, simple, and efficient way to determine the 3D orientation of single dipole emitters for applications ranging from cellular imaging to novel approaches in nanoscale confinement.

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