Chapter 5

3D-orientation determination of single molecules

Overview

One of the unique features of single molecule absorption and emission is their anisotropy due to the well-defined transition dipole for both processes, which allows the determination of the molecule’s orientation. While polarization-resolved techniques can commonly detect only a projection of the transition dipole, several methods have been proposed to determine the full three-dimensional orientation. Here, a newly developed detection scheme is presented in section 5.1, which allows for a shot-noise limited determination of the emission dipole orientation utilizing an annular mirror and a polarizing beam splitter combined with three detectors in a scanning confocal optical microscope. Monte-Carlo simulations are presented in section 5.2, evaluating the accuracy and the theoretical limits of the presented scheme for orientation determination. Moreover, the application of this methodology for the determination of the relative orientation of transition dipoles in crystalline poly(diacetylene) nanofibers in porous alumina is presented in section 5.3. A general discussion in section 5.4 will close this chapter.

5.1 Models and methods

The dipole emission $I_{Em}$ in a homogeneous medium can be described in spherical coordinates according to

$$I_{Em} = (\sin \theta \cos \phi \hat{e}_x + \sin \theta \sin \phi \hat{e}_y + \cos \theta \hat{e}_z)^2$$ (5.1)

with the polar angle $\theta$ and the azimuthal angle $\phi$. Figure 5.1 shows the corresponding plot, where the orientation of the emission dipole coincides with the $z$-axis. Interpreting the dipole emission as a probability distribution of the directions of emitted photons, it is obvious that most of the photons will be emitted close to the $x, y$-plane. Moreover, the probability of detecting a photon along the $z$-axis is essentially zero.

The main idea is to make use of the anisotropic nature of dipole emission. Therefore, the ratio of the integrated detection probabilities of two, sufficiently large areas is determined. These areas are defined by the detection scheme depicted in figure 5.2. The fluorescence light collected
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Figure 5.1: Normalized dipole emission plotted as a function of the polar angle $\theta$ and the azimuthal angle $\phi$ as defined in section 2.6. Here, the orientation of the dipole coincides with the z-axes. Whereas almost no emission occurs in the z-direction, the main contribution is emitted into the x- and y-plane.

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: light emitted in a hollow cone close to the rim of the microscope objective lens is reflected off the mirror while emission close to the optical axis is transmitted. The emission in the central cone is further divided into two orthogonally polarized contributions. All light components are focused onto avalanche photon diodes.

Figure 5.3 shows a more detailed view of the relation between dipole orientation and the fractions of light directed to the detectors. The area of the central cone is defined by $0 < \theta < \alpha_c$, where $\alpha_c$ is given by the annular beam splitter. The hollow cone is defined by $\alpha_c < \theta < \alpha_r$, where the cutoff angle $\alpha_r$ is given by the opening angle of the microscope objective. Here, by using an oil-immersion objective with $N.A. = 1.4$, $\alpha_c$ results in $\alpha_c = 67^\circ$. For the determination of $\theta$, two limiting cases can be discussed according to figure 5.3 b+c). For $\theta = 0^\circ$ only a small amount of light can be picked up by the central cone. In contrast, $\theta = 90^\circ$ will give a high contribution to the central cone, whereas the fraction of light emitted into the rim of

Figure 5.2: Diagram of the detection scheme for three-dimensional orientation determination of single molecules based on a confocal microscope. After excitation of the molecule, the emitted light is separated by an annular and a polarizing beam splitter into three fractions.
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Figure 5.3: a) The orientation of the emission dipole according to the optical axis \( z \) is given by the polar angle \( \theta \). The angle \( \alpha_c \) determines the inner cone of light, which can pass the annular beam splitter. The area between the angles \( \alpha_c \) and \( \alpha_r \) determine the fraction of light which is reflected by the annular beam splitter. b) First limiting case (\( \theta = 0^\circ \)): Almost no light is emitted into the inner cone. c) Second limiting case (\( \theta = 90^\circ \)): Most light is emitted into the inner cone.

The objective lens remains almost constant for both cases. The limiting cases will be of great importance during discussion of the accuracy of the proposed method.

Generally, the emission intensities in the central cone \( I_c \) and in the outer cone \( I_r \) can be expressed according to [26, 47],

\[
I_r(\theta, \phi) = 2I_{tot}(t, t+\tau) \left[ (A_r - A_c) + (B_r - B_c) \sin^2 \theta \right] \tag{5.2}
\]

\[
I_{0,c/90,c}(\theta, \phi) = I_{tot}(t, t+\tau) \left[ \frac{0, c}{90, c} \left[ A_r + B_r \sin^2 \theta \pm C_c \sin^2 \theta \cos 2\phi \right] \right] \tag{5.3}
\]

\[
I_c(\theta, \phi) = I_{0,c}(\theta, \phi) + I_{90,c}(\theta, \phi) = 2I_{tot}(t, t+\tau) \left[ A_c + B_c \sin^2 \theta \right] \tag{5.4}
\]

with \( I_{tot} \) as the total emitted intensity integrated over all directions in space for a given time period and the following variables

\[
A_{c,r} = \frac{1}{6} - \frac{1}{4} \cos \alpha_{c,r} + \frac{1}{12} \cos^3 \alpha_{c,r} \tag{5.5}
\]

\[
B_{c,r} = \frac{1}{8} \cos \alpha_{c,r} - \frac{1}{8} \cos^3 \alpha_{c,r} \tag{5.6}
\]

\[
C_{c,r} = \frac{7}{48} - \frac{1}{16} \cos \alpha_{c,r} - \frac{1}{16} \cos^2 \alpha_{c,r} - \frac{1}{48} \cos^3 \alpha_{c,r} \tag{5.7}
\]

which are fully defined by the detection angles \( \alpha_c \) and \( \alpha_r \).

With the common definition of the polarization \( P \) as \( P := (I_{90,c} - I_{0,c})/(I_{90,c} + I_{0,c}) \) and defining correspondingly an inclination \( N \) as \( N := (I_r - I_c)/(I_r + I_c) \), the polar angle \( \theta \) and the azimuthal angle \( \phi \) can be calculated according to

\[
\theta = \arcsin \sqrt{\frac{-2A_c - A_r + A_cN}{-2B_c - B_r + B_cN}} \tag{5.8}
\]
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Figure 5.4: a) $\Delta N$ (see equation 5.10) as a function of $\alpha_c$ using a fixed detection angle of $\alpha_r = 67^\circ$, which is defined by the numerical aperture of the microscope objective. $\Delta N$ defines the achievable contrast for determination of $\theta$ and reaches a maximum value for $\alpha_c = 44^\circ$. b) The center intensity $I_c$ ($\Delta$), the intensity in the rim $I_r$ (○) and the total detectable intensity $I_D$ (□) as a function of $\theta$ and using $I_{tot} = 1$. Detection angles of $\alpha_c = 44^\circ$ and $\alpha_r = 67^\circ$ were used for calculations according to equations 5.2-5.4.

and

$$\phi = \frac{1}{2} \arccos \left( -P \frac{A_c + B_c \sin^2 \theta}{C_c \sin^2 \theta} \right). \quad (5.9)$$

$N$ 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 to a range of $\theta$ and $\phi$ from $0^\circ$ to $90^\circ$.

In order to find the optimal value for $\alpha_c$, we consider the range of possible values $N(\alpha_c, \alpha_r, \theta) = (I_r - I_c)/(I_r + I_c)$. The angle $\alpha_c$ where the range $\Delta N$ has a maximum value can be found by calculating $d\Delta N/d\alpha_c = 0$. Figure 5.4 a) shows $\Delta N$ as a function of the cutoff angle $\alpha_c$ with a fixed rim angle $\alpha_r$. Using a rim angle of $\alpha_r = 67^\circ$ results in an optimal cutoff angle of $\alpha_c = 44^\circ$. For this cutoff angle, $N$ is defined between $N_{\min} = -0.08$ and $N_{\max} = 0.5$ and the contrast for determination of $\theta$ is maximized. Now, the detectable intensities $I_c(=I_{00,c} + I_{0,c})$, $I_r$ and $I_D(=I_c + I_r)$ can be plotted as a function of $\theta$ as shown in figure 5.4 b). In contrast to the rim intensity $I_r$, which is almost constant for each $\theta$-value, the center intensity $I_c$ increases with higher values of $\theta$. Note that the total detectable intensity $I_D$ is only a function of $\theta$ and varies between 15% and 25% of the light emitted by a single fluorophore.

Monte-Carlo simulations of orientation determination

The convolution of the collimated laser beam and the collection efficiency function can be approximated by a two-dimensional Gaussian in the $x$- and $y$-plane. The spatial intensity distribution $I(x,y)$ of a confocal microscope can be written as

$$I(x,y) = I_0 \exp \left( -2 \frac{(x-x_0)^2 + (y-y_0)^2}{w_0^2} \right), \quad (5.11)$$
where \( I_0 \) represents the maximum excitation intensity, \( x_0 \) and \( y_0 \) the center of the focus and \( w_0 \) the effective beam waist in the radial direction. Defining \( I_{tot} := I_0/Q \) as the total detectable intensity of the emitted light in the whole space, where \( Q = \sigma_{exc} \phi_f \kappa \) represents the product of the excitation cross section of the fluorescent molecules \( \sigma_{exc} \), their fluorescence quantum yield \( \phi_f \), and the setup-specific efficiency of detection of fluorescence \( \kappa \) [112], the detectable intensity \( I_D \) through the objective can be written according to

\[
I_D = I_r + I_c = 2 \cdot Q (A_r + B_r \sin^2 \theta) I_{tot} \exp \left( -2 \frac{(x-x_0)^2 + (y-y_0)^2}{w_0^2} \right). \tag{5.12}
\]

In the following, \( Q \) is set to unity without loss of generality. The shot-noise nature of dipole emission is taken into account using a Poisson distribution, where the probability \( P \) of detecting \( k \) photons with an expectation value of \( \lambda \) is given by

\[
P(X = k) = \frac{\lambda^k e^{-\lambda}}{k!}, \tag{5.13}
\]

with \( k = 0,1,2,... \) and \( \lambda > 0 \). Furthermore, the Poisson distribution is characterized by \( \lambda = \sigma^2 \), where \( \sigma \) represents the standard deviation. The orientation, position (if applicable), and number of detected photons subject to a Poisson distribution are stochastically generated by Monte-Carlo methods implemented in Igor (Wavemetrics). Three types of simulations were carried out, namely simulations of orientation-resolved images (\textbf{type A}) and simulations of orientation-resolved transients (\textbf{type B} and \textbf{type C}).

For simulations of \textbf{type A} 8 molecules were placed at randomly distributed positions defined by \( x_{0,i} \) and \( y_{0,i} \) within an area of \( 5 \times 5 \mu m \) (128 \times 128 pixels). Coordinate \( z \) was set to zero. The azimuthal angles \( \phi_{0,i} \) and the polar angles \( \theta_{0,i} \), which determine the individual orientation of the emission dipole, were randomly distributed following a uniform distribution for azimuthal angles and a sinusoidal distribution for polar angles corresponding to the surface area element in polar coordinates. The intensities \( I_{0,c} \), \( I_{0,r} \), and \( I_r \) were calculated for each pixel by using equations 5.2 and 5.3 with \( I_{tot} = 2000 \) photons, \( w_0 = 0.25 \mu m \), \( \alpha_r = 67^\circ \) and \( \alpha_c = 44^\circ \). Every calculated intensity value was used as a mean \( \lambda \) in equation 5.13 to stochastically generate a new intensity following Poisson distribution. The obtained image is just the sum of the intensities of the eight molecules. Finally, Poisson-distributed background luminescence of 5 photons per pixel was added pixel-by-pixel to \( I_{0,c} \), \( I_{0,r} \), and \( I_r \).

For simulations of \textbf{type B} and \textbf{type C} one single molecule at a time was placed in the center of the intensity distribution, i.e. \( x = y = x_0 = y_0 = 0 \), with a fixed orientation defined by the azimuthal angle \( \phi_0 \) and the polar angle \( \theta_0 \). To mimic a time-dependent experiment, the procedure of adding noise to the beforehand calculated intensity ratios was carried out several thousand times. For simulations of \textbf{type B}, the total number of emitted photons per second was set to half a million. Assuming a time resolution of 2ms, the corresponding total intensity \( I_{tot} \) was set to \( I_{tot} = 1000 \) photons for each bin. The duration of the simulated transient time trace was 100 s.

As it was shown in equation 5.12, the detectable intensity \( I_D \) is a function of the polar angle \( \theta \). Instead of using \( I_{tot} = \text{const.} \) as for simulations of \textbf{type B}, simulations of \textbf{type C} follow a different approach. Here, the probability of detecting a single photon in one of the three
detectors is calculated as a function of the orientation using $I_{tot} = 1$. Poissonian-distributed noise is added to each probability. This addition of noise is iterated and the cumulative number of detected photons is stored, until a beforehand defined number of photons $I_{No}$ is reached for this bin. The advantage of this approach is twofold: the $\theta$-dependence of the detection probability is circumvented, and the number of detected photons per bin can be attributed to different binwidths. For simulations of type C, different numbers of photons per bin [either $I_{No} = 500$ or $I_{No,m} = 100 \cdot 2^m/2$, with $m = 0, 1, 2...$ was used] and different initial orientations $[\phi_{0,i}(\theta_{0,i}) = 0^\circ, 5^\circ, ..., 90^\circ]$ were used. The overall number of simulated bins was set to 200.

Additionally, for simulations of type B the addition of background photons was slightly modified. In contrast to the anisotropic nature of dipole emission, the nature of background emission is considered being isotropic. In this case the ratio between the detected background intensity in the center and the overall detected intensity is given by the ratio of the solid angles

$$\Delta \Omega = \frac{2\pi (1 - \cos \alpha_c)}{2\pi (1 - \cos \alpha_r)} = \frac{2\pi (1 - \cos 44^\circ)}{2\pi (1 - \cos 67^\circ)} = 0.46. \quad (5.14)$$

In other words, 46% of the isotropic background intensity will be detected in the center (and therefore 23% in each of the two detectors) and 54% will be detected in the rim.

Note that this result is slightly different from the case of superposition of randomly oriented dipoles. This would lead to a determination of $\theta = 60^\circ$ and $\phi = 45^\circ$ (magic-angle condition) according to the uniform distribution for azimuthal angles and the sinusoidal distribution for polar angles corresponding to the surface area element in polar coordinates. In this case, using $\alpha_c = 67^\circ$ and $\alpha_r = 44^\circ$ leads to $I_{rim}(\theta = 60^\circ) \approx I_c(\theta = 60^\circ)$. Therefore, half of the detectable background intensity $I_{Bg}$ would be detected by the rim detector and the other half would be directed to the two detectors for the central beam. For sake of simplicity, if not stated otherwise, no background was added to simulations of type C.

Using these intensity patterns of simulations of type B and type C allows for re-calculating the orientations of the molecules bin by bin as a function of the beforehand defined azimuthal angles $\phi_{0,i}$, the polar angles $\theta_{0,i}$, the excitation intensities defined by $I_{No,m}$, and the detectable background intensity $I_{Bg}$ in order to study the influence of the shot-noise nature to orientation determination. The distributions of re-calculated angles can be characterized by their mean and their standard-deviation.

## 5.2 Results of simulations

Figure 5.5 shows a simulation of type A after placing a set of molecules with randomly chosen orientation into a scanning area. The three detectable intensity images ($I_{0,c}$, $I_{90,c}$ and $I_r$) and their sum $I_{sum} \equiv I_D$ show the typical Gaussian intensity distribution reflecting the point spread function. The intensity images of $I_{0,c}$ and $I_{90,c}$ show different maximum intensities for the molecules, whereas the images of $I_r$ and $I_{sum}$ look rather uniform. Nevertheless, it is obvious that different orientations of the molecules lead to different detectable intensities, which is a direct result of the anisotropy of dipole emission. After setting a appropriate intensity threshold value, the calculated intensity patterns were further used to re-calculate the azimuthal angles $\phi_i$ and the polar angles $\theta_i$ pixel by pixel according to the equations 5.9 and 5.8. Each molecule is
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labelled with the initially used angles $\phi_{0,i}$ and $\theta_{0,i}$ for comparison. The averaged reconstructed values for each molecule are well in line with those initially set. However, there are several distinctive features: 1) not every molecule shows an uniform angular pattern, 2) especially molecules with a high $\theta$-value show a lot of missing pixels (→ white pixels), and 3) the fringes of some molecules show mean values different from those of the inner part. In contrast, figure

Figure 5.5: Simulations of type A of 8 randomly oriented and randomly distributed molecules on an area of 5 µm by 5 µm. $I_{0,c}$, $I_{90,c}$, and $I_r$ show the pseudo color-coded, calculated fluorescence intensity as it would be detected after scanning the area with a confocal microscope. $I_{\text{sum}}$ represents the sum of the previous intensities. After setting an appropriate threshold, the intensities were used to re-calculate the orientation patterns $\theta$ and $\phi$ pixel by pixel. Note that for all calculated intensities Poissonian noise was added.
5.6 shows angular patterns of $\theta$ and $\phi$, where the calculated intensity patterns (data not shown) were not Poissonian distributed pixel by pixel afterwards. Here, each molecule shows an uniform and perfect representation of the initial orientation, as long as no overlap of the molecules occurs. This possible overlap, as shown in figure 5.6, is a very interesting feature. As long as the orientations of those molecules are sufficiently different, the calculated angular patterns allow an easier spatial separation than the intensity pattern. Note that the overall emitted intensity of one single molecule is a function of $\theta$. The molecules with the highest values of $\theta$ dominate the other ones according to figure 5.4 b).

Comparing the angular patterns of figures 5.5 and 5.6 reveals that the shot-noise nature of photon emission is the main limiting factor for three-dimensional orientation determination. Generally speaking, every orientation of a fluorophore is defined by a particular relation between the intensities $I_{0,c}$, $I_{00,c}$ and $I_r$. However, due to the shot-noise nature of photon emission, only a probability distribution of detectable intensities can be given. After adding the Poisson-distributed noise to the detectable intensities, the initial particular relation between the intensities is changed. Re-calculation of the orientation will lead to apparently altered values of $\theta$ and $\phi$, as long as there is still an orientation defined for the new relation of the intensities. Especially for values of $\theta, \phi \approx 0^\circ$ or $\theta, \phi \approx 90^\circ$ ($0^\circ \leq \phi, \theta \leq 90^\circ$), the set of valid intensity-relations is limited. This is the reason for the ”missing” pixels in figure 5.5. The calculation of $\phi$ is based on the calculation of $\theta$; missing pixels in the $\theta$ pattern will automatically lead to missing pixels in the $\phi$ pattern. To solve this problem, pixels from forbidden intensity-relations can be filled either with $\theta, \phi = 0^\circ$ or $\theta, \phi = 90^\circ$ according to the nearest valid relation. The overall number of valid relations is a function of the detected intensities: if the detected intensities (and therefore the overall number of emitted photons) is high, the Poissonian nature of photon emission becomes less relevant.

Simulations of type B were performed in order to elaborate the effect of the Poissonian photon statistics in transient time traces. After fixing the orientation of an emission dipole to $\theta_0 = 60^\circ$ and $\phi_0 = 25^\circ$ the three detectable intensities $I_{0,c}$, $I_{00,c}$ and $I_r$ were calculated for each
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Figure 5.7: Simulation of transient time traces for orientation determination of the emission dipole of single molecules. The initial orientation was fixed to $\theta_0 = 60^\circ$ and $\phi_0 = 25^\circ$. a) Detectable time-traces using $I_{\text{tot}} = 1000$ photons per 2 ms: $I_{\text{tot}}$ (-$\triangle$, spare symbols every 25th data point), $I_{\phi,0}$ (-$\square$), and $I_{r}$ (-$\circ$). b+c) Re-calculated orientations of $\phi$ and $\theta$. Note that the lines are interrupted (as indicated by the arrows). d) Histograms of the re-calculated angles $\phi$ (red, left) and $\theta$ (blue, right)

bin with a width of 2 ms using $I_{\text{tot}} = 1000$ photons per bin. Figure 5.7 a) shows sections of the transient time traces. The fluctuations in the intensities are only caused by the shot-noise nature of photon emission. Note that the time-resolution is 2 ms. During this time the total detected intensity of each bin is about 210 photons. In figure 5.7 a+b) this number of detected photons is sufficient to re-calculate $\phi$ and $\theta$ with high reliability. Nevertheless, it should be noted that the traces of the recalculated angles are interrupted, were the relation of the three detected intensities exceeds the defined range due to shot noise. The histograms of $\phi$ and $\theta$ re-calculated from the full intensity traces are shown in figure 5.7 d). Due to the non-linearity of the equations for calculating the angles, the finding of the non-symmetric angle distributions is not unexpected. The re-calculated mean values ($\theta_{\text{avg}} = 59.9^\circ \pm 9.3^\circ$ and $\phi_{\text{avg}} = 24.5^\circ \pm 4.1^\circ$) are in almost perfect agreement with the initially set values of $\theta_0 = 60^\circ$ and $\phi_0 = 25^\circ$. However, the initially chosen orientation is a "friendly" one; the angles are neither close to 0° nor to 90°. The problems arising from orientations close to 0 and 90°, the influence of the number of detected photons per bin on the reliability of three-dimensional orientation determination, and the most important question what conclusions can be drawn from the simulations for the experiments will be discussed in the following. Simulations of type C were performed for this purpose. As described in section 5.1, simulations of type C feature a constant number of detected photons per bin instead of defining a constant number of emitted photons by $I_{\text{tot}}$. The
number of detected photons is composed by the probabilities of detecting photons in one of the three channels representing $I_{0,c}$, $I_{90,c}$ and $I_r$. Figure 5.8 shows such a simulation with 500 photons per bin and a duration of simulation of 200 bins. The initial orientation was varied from 0° to 90° in steps of 5° for all angles $\theta_{0,i}$ and $\phi_{0,i}$. Note that bins featuring non-valid ratios of the intensities are discarded and not assigned to a certain value. Figure 5.8 a+b) show the pseudo color-coded re-calculated mean orientation of all bins of one orientation trace as a function of the initially set values of $\theta_{0,i}$ and $\phi_{0,i}$. The initial orientation was varied from 0° to 90° in steps of 5° for all angles $\theta_{0,i}$ and $\phi_{0,i}$. Note that bins featuring non-valid ratios of the intensities are discarded and not assigned to a certain value. Figure 5.8 a+b) show the pseudo color-coded re-calculated mean orientation of all bins of one orientation trace as a function of the initially set values of $\theta_{0,i}$ and $\phi_{0,i}$. In the case of the re-calculated $\theta$-values, they are well in line with the initially set values as long as $\theta \neq 0°$ and $\theta \neq 90°$. According to the arc sine and the square root in equation 5.8, $\theta$ is only defined between 0° and 90°. If the shot-noise nature of photon emission causes new relations of the three detected intensities which differ from the initial ones, only those contribute to the re-calculation which are still valid. This is the reason why the re-calculated average $\theta$-value is slightly higher for $\theta_{0,0}$- and slightly lower for $\theta_{0,90}$- than expected. The re-calculated $\phi$-values show a different behavior. If the initial $\theta_{0}$-value is smaller than 10°, every initial $\phi_{0,i}$-value leads to a re-calculated value with a averaged value of around $\phi \simeq 45°$. To explain this finding let us take the limit of equation 5.9 according to

$$\phi = \frac{1}{2} \arccos \left( \frac{-P A_c + R_c \sin^2 \theta}{C_c \sin^2 \theta} \right) \rightarrow \phi = \lim_{\theta \to 0°} \left( \frac{1}{2} \arccos (0) \right) = 45°. \quad (5.15)$$

Small values of $\theta$ result in almost indeterminable $\phi$-values, which is obvious taking into consideration that small $\theta$ means an orientation almost parallel to the optical z-axis.

The considerations for initial $\theta$-values around 0° or 90°, as discussed above, do also hold for $\phi$-values around 0° or 90°. Nevertheless, re-calculation of $\phi$ benefits from large values of $\theta$: if $\theta_{0,i}$ is larger than 80°, the re-calculated $\phi$-value maches almost perfectly the initial $\phi_0$-value. Figure 5.8 c+d) represent the standard deviation of the re-calculated angles as a function of their initial values. For the re-calculated $\theta$-values, the standard deviation is in any case smaller than 10°, which is a reasonable value keeping in mind that the overall number of detected photons was restricted to only 500 photons per bin. With increasing initial values of $\theta_{0,i}$, the standard deviation increases from 2.5° to around 8°, due to the non-linearity of the equations for calculating the orientation. For small $\theta_{0,i}$ angles the determination of $\phi$ leads to apparent values, as can be seen in figure 5.8 b). It is not surprising that the standard deviation is indeed large (around 15°). With increasing values of $\theta_{0,i}$, the standard deviation drops below 5°, which makes the detection scheme very suitable for determination of the azimuthal angle. Moreover, one should keep in mind that, assuming a completely random distribution of dipole orientations, the distribution of the polar angle $\theta$ scales with its sine and orientations with small angles of $\theta$ are rare.

Figure 5.8 e+f) serves the purpose to estimate how many of the 200 calculated bins are valid for re-calculation of either $\theta$ or $\phi$. Both images support the previous findings: values of $\theta_{0,i}$ close to 0° or 90° hamper the re-calculation of those initial values. In fact, by using these values almost 60% of the calculated bins contain non-valid intensity ratios. The re-calculation of $\phi$-values is even more difficult due to the fact that the calculation of $\phi$-values is based on the calculation of $\theta$-values. For this reason, re-calculation of $\phi$ suffers from $\phi$-values close to either 0° or 90° and, additionally, from $\theta$-values close to either 0° or 90°. Especially for large
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Figure 5.8: Simulations of type C for three-dimensional orientation determination. Different properties are plotted using pseudo-coloring as a function of the initial orientation defined by the values of $\theta_0$, $\phi_0$, $\omega_0$. Undefined ratios of the calculated detectable intensities were not fixed. 

- a+b) Re-calculation of $\theta$ and $\phi$ from the simulated detectable intensity traces.
- c+d) Standard deviation of the re-calculated angle-traces.
- e+f) Percentage of non-valid bins for re-calculation of the orientation out of the simulated intensity traces.

Values of $\theta_0$, $\phi_0$, and $\omega_0$, there is almost no valid bin. This has to be taken into account if the apparent small standard deviations in figure 5.8 d) are considered.

As discussed previously, the non-valid ratios of detected (simulated) intensities can be set to
the next valid ones. As a result, the re-calculated angles $\theta$ or $\phi$ are either fixed to $\theta (\phi) = 0^\circ$ or $\theta (\phi) = 90^\circ$. Thus, the number of non-valid bins is zero. Figure 5.9 shows simulations using the same parameters as discussed before but with fixing non-valid ratios. As seen in figure 5.9 a+b), the averaged re-calculated orientation at the edge is closer to the initial orientation than in figure 5.8. Especially re-calculation of $\phi$ benefits from this fixing of the angles at the edges as can be concluded from the reduced standard deviation in figure 5.9 d), whereas the standard deviation for the re-calculated $\theta$-values remains almost unchanged. In conclusion, 500 detected photons are sufficient for re-calculating the orientation, as long as the initial orientation is not close to values of either $0^\circ$ or $90^\circ$. Therefore, in experiments attention should be paid to both angles. If, for example, a value of $\theta \simeq 5^\circ$ was calculated, calculation of $\phi$ will lead to $\phi = 45^\circ$ absolutely independend of the real $\phi$-value.

In order to check the influence of the number of detected photons per bin on the accuracy of orientation determination, the following simulations are performed. Instead of going through all possible configurations of $\theta_{0,i}$ and $\phi_{0,i}$, the azimuthal angle $\phi_0$ was fixed to $\phi_0 = 45^\circ$ for each case. Only the initial values of $\theta_{0,i}$ were changed using stepwidths of $5^\circ$. As an additional parameter, the number of detected photons per bin was changed according to $I_{N_0,m} = 100 \cdot 2^m / 2$. 

Figure 5.9: Simulations of type C for three-dimensional orientation determination. Different properties are plotted using pseudo-coloring as a function of the initial orientation defined by the values of $\theta_{0,i}$ and $\phi_{0,i}$. Non-valid ratios of the calculated detectable intensities were fixed to the nearest valid ratio. a+b) Re-calculation of $\theta$ and $\phi$ from the simulated detectable intensity traces. c+d) Standard deviation of the re-calculated angle-traces.
5.2. Results of simulations

Figure 5.10: Simulations of type C for three-dimensional orientation determination. a) the re-calculated values of $\theta$ and b) the corresponding standard deviations are plotted as a function of the initial orientation $\theta_0$, and the number of detected photons per bin $I_{N_0,\text{m}}$ (logarithmic scale). No background noise was added to the simulations, ill-defined intensity ratios were fixed to valid ones.

with $m = 0, 1, 2, ..., 12$. The overall number of bins was set to 200. Figure 5.10 a) shows the averaged value of the re-calculated values of $\theta$ as a function of the initial $\theta_0$ and $I_{N_0,\text{m}}$. Again, all non valid-ratios were fixed to the next valid ones. Whereas the re-calculated mean values in figure 5.10 a) show only slight differences with an increasing number of photons per bin, the standard deviation drops significantly from around $\Delta \theta_0 = 7^\circ ... 15^\circ$ for $I_{N_0,0} = 100$ photons per bin to $\Delta \theta_0 = 0^\circ ... 6^\circ$ for $I_{N_0,12} = 4525$ photons per bin. This finding may indicate the potential time-resolution of the determination of the orientation of single molecules. Let us assume a detectable count rate of 200 kHz for an immobilized molecule with an arbitrary orientation of the emission dipole. Even with 100 photons per bin, the re-calculated mean $\theta$-value reproduces the initial orientation with sufficient accuracy. Dividing 200000 photons per second with 100 photons per bin equals 2000 bins per second corresponding to a time resolution of 500 $\mu$s. The more photons are acquired for re-calculating the orientations, the higher the accuracy that can be obtained.

It should be explicitly mentioned that the orientation determination from experimental data is a so-called inverse problem. Even if the model applied for the MC simulations is straightforward, the interpretation of the re-calculated orientation using experimental data is rather complicated. In fact, there is no fitting function which could be used to fit the probability distributions obtained by re-calculating the orientation for a number of bins of one emission dipole. Moreover, as discussed above, re-calculating of $\phi$-values for orientations with small $\theta$-values will result in $\phi_0 \simeq 45^\circ$.

From an experimental point of view, it is of interest with which probability a measured intensity distribution (and therefore a calculated orientation) corresponds to the actual orientation of the emission dipole. This issue is explored by the Bayes theorem [3], which relates the probability
of an event \( A \) conditional on another event \( B \) to the (in generally different) probability of \( B \) conditional on \( A \). Bayes claimed that the \textit{a posteriori} probability \( P(A \mid B) \) of detecting event \( A \) under condition of event \( B \) is related to the likelihood function \( P(B \mid A) \) for \( A \) given \( B \) by a factor containing the \textit{a priori} probability of \( A \) namely \( P(A) \) and the one of \( B \) namely \( P(B) \) according to

\[
P(A \mid B) = \frac{P(B \mid A) \cdot P(A)}{P(B)}. \tag{5.16}
\]

Let us now relate this description to the problem of orientation determination. The \textit{a priori} probability \( P(A) \) describes the probability of appearance of each orientation. As mentioned in section 5.1, these \textit{a priori} probabilities follow a uniform distribution for azimuthal angles \( \phi \) and a sinusoidal distribution for polar angles \( \theta \) corresponding to the surface area element in polar coordinates for random orientations. For example, \( P(A) \) reads \( P(A(\phi = 40^\circ)) = 1/90 \) for the distribution of the azimuthal angles using increments of one degree. The second \textit{a priori} probability \( P(B) \) acts as a normalizing constant and can be determined using the MC-simulations. Here, \( P(B) \) is the sum of the probabilities (as for example shown in figure 5.7 d)) of detecting a fixed orientation \( \chi \) for each possible initial orientation. The likelihood function \( P(B \mid A) \) is determined by the probability of re-calculating \( \chi_{re} \) by using \( \chi \) as the initial orientation for the MC-simulation. Now, \( P(B \mid A) \) can be calculated for each detected orientation. Superimposing the calculations of \( P(B \mid A) \) for different detected orientations will lead to a probability distribution, giving the possibility to calculate the initial orientation out of some detected orientations. Note that in many systems of interest \( P(A) \) is precisely not fully randomly distributed.

The last point in this section adresses the influence of the isotropic background on the re-calculation of a given orientation. Again, only the influence on the re-calculation of the \( \theta \)-values was studied. Whereas the number of detected photons per bin was fixed to \( I_{No} = 400 \) photons, the composition of this number was changed during simulation. At the beginning, all photons per bin were attributed to the fluorescent molecule of interest. Then, using stepwidths of 10%, a given percentage of photons per bin was attributed to isotropic background fluorescence. For example, by using a noise level of 10%, 360 photons are attributed to the fluorophore and 40 photons to background noise. As shown in figure 5.11 a), the noise level was varied between 0% and 100%. Under experimental conditions, a noise level of less than 10% can be achieved in many cases. With this level, the re-calculated values are close to the initial ones. If the noise level is further increased, the distribution of photons into the three detectors will be dominated by the background photons. Thereby, the initial orientation can not be re-calculated and leads in any case to \( \theta \approx 60^\circ \) as discussed in section 5.1.

The standard deviation of the re-calculated \( \theta \)-values is shown in figure 5.11 b). The standard deviation is almost constant with an increasing level of noise and can not be used as an additional criterion to distinguish between a low or a high level of noise. In conclusion, Monte-Carlo simulations of the orientation determination offer an important tool to explore the possibilities and the limits of the proposed detection scheme. It was shown that, depending on the desired accuracy, a high time resolution can be obtained. However, the method is limited by the shot-noise nature of dipole emission. Especially dipole orientations close to \( \theta(\phi) = 0^\circ \) or \( \theta(\phi) = 90^\circ \) cannot be determined with high accuracy. Moreover, it should be mentioned...
5.3 Experimental results

Fluorescent latex beads

As discussed above, the alignment and correction procedures for three-dimensional orientation of the emission dipole are far from being trivial. In order to develop an appropriate correction, fluorescent latex beads (diameter 100 nm, Sigma-Aldrich) were used, which were immobilized on the cover glass surface by drying an aqueous corresponding suspension of the beads. The concentration of the beads was chosen in such a way that after immobilization the mean inter-bead distance was large enough to clearly separate single beads with the confocal microscope. For these experiments, a water-immersion objective was used, featuring a beam diameter of 7 mm in the path of detection. A home-made mirror with an elliptical aperture was used to separate the inner detection beam from the outer beam. The diameter of the inner beam that could pass the mirror was about 3 mm. The overall detection angle of the water-immersion objective is given by its numerical aperture $NA = 1.2$, which results in a detection angle of $\alpha_e = 64^\circ$ in water assuming an refractive index of $n = 1.33$. Geometrical considerations show that (taking the radii of the inner and the outer beam of 3.5 mm and 1.5 mm, respectively)
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Figure 5.12: Experimental realization of the detection scheme for determining the three-dimensional orientation of emission dipoles. The incoming fluorescent light is at first separated by an annular mirror and at second by a polarizing beam splitter.

\[
\frac{3.5 \text{ mm}}{\tan 64^\circ} = \frac{1.5 \text{ mm}}{\tan \alpha_{c,1}} \tag{5.17}
\]

results in \( \alpha_{c,1} = 41^\circ \), which is close to the ideal angle of \( \alpha_{c,0} = 44^\circ \) theoretically derived in section 5.1. A polarization beam splitter was used for further separation of the inner detection beam. The experimental realization of the proposed detection scheme is shown in figure 5.12. To match the requirements of the water-immersion objective, the beads were covered with water. No desorption of latex beads was detected. 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 500 nm long pass filter was used to filter out the remaining laser light. An area of 5 µm by 5 µm was raster-scanned using the piezo-driven scanning stage with a resolution of 128 by 128 pixel with an integration time of 2 ms per pixel. Three intensity images were obtained (data not shown, see section 5.2 for comparison) for each scan representing \( I_0, c \), \( I_{90, c} \), and \( I_r \), which were used to calculate pixel by pixel the \( \theta \)- and \( \phi \)-values according to the equations 5.9 and 5.8. Figure 5.13 a) and b) show the corresponding values of \( \theta \) and \( \phi \) after applying an intensity threshold of 50 photons per pixel and appropriate correction. Each fluorescent latex bead contains a large number of randomly orientated fluorophores. In this case, as discussed in section 5.1, the expected average orientation values are known and can be used for correction as follows. To correct for the calculation of \( \phi \), the weighting of photons detected in one of the two detection channels for \( I_c \) (\( I_{0, c} \) and \( I_{90, c} \)) can be changed in such a way that the histogram of \( \phi \)-values shown in figure 5.13 d) is centered at \( \phi_{\text{avg}} = 45^\circ \pm 4^\circ \). Here, the detected number of photons in the detector of \( I_{90, c} \) was multiplied with 1.1 (in the following written as \( I_{90, c} = 1.1 \cdot I_{90, c} \) ). To correct for the calculation of \( \theta \), a different approach was used. The ratio of the inner and the outer intensity (\( I_c \) and \( I_r \)) is determined by the diameter of the elliptical aperture of the mirror. Therefore, instead of weighting the number of detected photons, the
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Figure 5.13: Calculated orientation patterns for five fluorescent latex beads immobilized on a cover glass surface. Three recorded intensity images (data not shown) were used for determination of a) $\theta$-values and b) $\phi$-values after applying a certain intensity threshold. The average angle of each single bead is appended. Histograms (pixel by pixel) of both images showing the appearance of c) $\theta$-values and d) $\phi$-values.

cutoff angle $\alpha_c$, which is used for the calculations, was set to $\alpha_c = 41^\circ$ so that the average $\theta$-value of the histogram is $\theta_{\text{avg}} = 59^\circ \pm 10^\circ$. Using this approach, the initial calculated center angle $\alpha_{c,1} = 41^\circ$ was verified. According to the relationship of the three detectable intensities, correcting the $\theta$-value will effect the $\phi$-value and vice versa so careful tuning is necessary. By setting the (sum-)intensity threshold for the calculation of the orientation to 50 photons, the calculated patterns in figure 5.13 a) and b) do not show fringes with different color coding than the center of each bead as shown in figure 5.5. This indicates that the threshold value is sufficient to suppress the influence of the background luminescence and the shot-noise. Moreover, even if the detected intensity per pixel is increasing from the rim to the center of each bead, no further enhancement of the accuracy of the calculation according to the expected values is visible.

In order to evaluate the behavior of a single bead, the focus was placed in the center of the first bead (as indicated in figure 5.13). Time traces of the three detectable intensities were recorded for several seconds with a time resolution of 100 µs. The recorded time traces were further binned to a time resolution of 1 ms. The overall count rate was around 150 kHz, so each bin contained about 150 photons, distributed among the three detection channels. The calculation of the orientation was performed using the corrections determined above ($\alpha_c = 41^\circ$ and $I_{90,c} = 1.1 \cdot I_{90,c}$). Figure 5.14 a) and c) shows sections of the complete calculated $\theta$- and $\phi$-
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Figure 5.14: Calculated orientation time-traces of a single bead immobilized on a cover glass. The shutter of the laser opened 25 ms after starting the recording. The binwidth of the detected intensity traces was set to 1 ms. a) calculated \( \theta \)-values as a function of time (small cutout of the whole trace), b) corresponding histogram (normalized) of calculated \( \theta \)-values. The solid black line represents a histogram obtained from MC-simulation using the values determined by the experiment as initial seeds. c+d) orientation time-traces and corresponding histogram for \( \phi \)-values.

traces as a function of time. Note that the laser shutter opened 25 ms after starting the recording and that the traces are interrupted. These voids correspond to ill-defined intensity ratios that do not allow attributing angles to these ratios, as discussed in section 5.2. The average \( \theta \)- and \( \phi \)-values were determined to \( \theta = 57^\circ \pm 10^\circ \) and \( \phi = 45^\circ \pm 4^\circ \) respectively. These values are in perfect agreement with the values determined by averaging over the single bead as shown in figure 5.13. The normalized histograms of the calculated \( \theta \) and \( \phi \)-values are shown in figure 5.14 b) and d) with a scalewidth of 2\(^\circ\). The histograms are rather smooth. In addition to the histogram of the calculated experimental data, a black line in both figures represents the normalized histogram of re-calculated \( \theta \)- or \( \phi \)-values obtained from MC-simulations using \( \theta_0 = 57^\circ \) and \( \phi_0 = 45^\circ \) as initial values. Additionally, \( \alpha_c \) was set to 41\(^\circ\) and each bin with a width of 1 ms was calculated using \( I_{tot} = 750 \) photons per bin. Taking into account that, according to figure 5.4, only around 20\% of the overall number of emitted photons can be detected, this corresponds to the number of 150 photons per bin as detected in the experimental case. No further background was added. The normalized histograms of the experimental data coincide almost perfectly with those obtained by MC-simulations, because the applied model for calculation is the same in both cases. This is indicative of proper alignment and correction for the experimental data. However, this coincidence leads to additional conclusions. According to
the literature, the emission dipole of a fluorophore placed directly at the interface of two media with different refractive indices (here: water and glass), exhibits a significantly modified emission profile as compared to a dipole in a homogeneous medium [22]. Nevertheless, at least after applying the correction, the simplified model of the undistorted emission dipole allows recovering the theoretical predicted orientation ($\theta = 60^\circ$ and $\phi = 45^\circ$ for a superposition of a large number of fluorophores as it is the case for a fluorescent latex bead) with high accuracy, indicating that most of the fluorophores in the latex bead are sufficiently far away from the interface. Moreover, the coincidence, even without applying background luminescence to the simulated data, evidences the very high obtainable signal-to-noise ratio.

**Perylene monoimide**

Even if some of the characteristics of the proposed method for orientation determination can be determined using fluorescent latex beads, the real challenge is the determination of the orientation of the emission dipole of a single fluorophore. For this purpose, different samples were prepared by spin-casting a polymer film from a co-solution of poly(methyl-methacrylate) (PMMA) and perylene monoimide (PMI) in toluene onto a microscope cover slip. The ratio of PMMA/toluene was 5 g/l. The resulting approximately 50 nm-thick polymer film contains the PMI chromophores in arbitrary orientations. Circularly polarized light with a power of 5 µW was used for excitation, and the sample was covered by immersion oil to fulfill the requirement of a homogeneous refractive index in the vicinity of the dipole using an oil-immersion objective for excitation/detection. An area of 5 µm by 5 µm was raster-scanned (128 by 128 pixels) with a dwell time of 2 ms. Figure 5.15 a-d) shows the three detected intensities $I_{0,c}$, $I_{90,c}$ and $I_{r}$ and their calculated sum ($I_{sum}$) thus obtained. A comparison of individual spots in figure 5.15 a) and figure 5.15 b) reveals differences in the measured intensities, which can be directly attributed to different azimuthal angles. As described in the previous section, careful corrections are necessary for calculating the orientation patterns. Here, an oil-immersion objective (NA = 1.4, 100x magnification) was used for the experiments with PMI. In contrast to the water-immersion objective, the oil-immersion objective features a smaller detectable beam diameter of around 5 mm and a detection angle of $\alpha_c = 67^\circ$. Using the mirror with an ellipsoidal aperture projected as a circle 3 mm in diameter leads to $\alpha_c = 55^\circ$. Nevertheless, corrections using immobilized latex beads (data not shown) revealed that the effective cut-out angle is $\alpha_c = 41^\circ$. This relatively large discrepancy can be explained by the small beam diameter caused by the pupil of the oil immersion objective in conjunction with the suboptimal quality of the annular mirror (see figure 5.12). Potential improvements will be discussed in section 6. The detected number of photons in the detector of $I_{90,c}$ had to be multiplied with 1.15. Applying these correction factors obtained from images of fluorescent latex beads in a first approximation, the scanned images of PMI can be used to refine the correction factors as long as some assumptions are fulfilled. If a sufficiently large number of molecules is imaged, and a random orientation of the molecules is assumed, the expected averaged orientation will be again $\theta_{avg} = 60^\circ$ and $\phi_{avg} = 45^\circ$. Based on these considerations, $\alpha_c = 43^\circ$ and $I_{90,c} = 1.2 \cdot I_{90,c}$ were derived. The orientation was calculated for each pixel above a fluorescence intensity threshold of 40 photons. Figure 5.15 e+f) shows the calculated $\theta$- and $\phi$- pattern. Note that
Figure 5.15: Single Perylenediimide molecules dispersed in a PMMA film. a-d) $I_{0,c}$, $I_{90,c}$ and $I_r$ show the pseudo color-coded, detected fluorescence intensity obtained by scanning the sample. $I_{sum}$ represents the sum of the previous intensities. e-f) after setting an appropriate threshold, the intensities were used to calculate the orientation patterns $\theta$ and $\phi$ pixel by pixel. Additionally, four molecules were selected for further recording of stationary time traces as indicated in d), e), and f).

non-valid ratios were converted to the closest valid ratios. The different colors of the various spots representing single dye molecules reflect their arbitrary orientations. The colors of the pixels in individual spots vary due to shot noise. The low incidence of spots with a low $\theta$, representing molecules oriented close to the optical axis, can be understood keeping in mind that the surface area element in polar coordinates scales with the sine of the polar angle. Com-
5.3. Experimental results

Figure 5.16: Normalized histograms of the calculated angles a) $\theta$ (blue) and b) $\phi$ (red). The pixel by pixel histograms were calculated using figure 5.15 e+f). The solid black lines represent the expected probability distributions of $\theta$- and $\phi$-values, assuming a sinusoidal distribution of the polar angles $\theta$ and an equal distribution of the azimuthal angles $\phi$.

Comparing the intensity image in figure 5.15 d) with the $\theta$-pattern of figure 5.15 e) one notices that molecules oriented close to the optical axis show a rather weak fluorescence. This is because the component of the exciting electrical field in the focus parallel to the optical axis is small compared to the perpendicular component and hence, according to figure 5.4 b), the detectable emission intensity is smaller than for large values of $\theta$.

The probability distributions of calculated $\theta$- and $\phi$-pixels are shown in figure 5.16. In addition, the theoretically expected distribution is plotted within the figures. The distributions resemble those features expected for arbitrarily oriented molecules: The azimuthal angle $\phi$ is evenly distributed, and the polar angle $\theta$ shows an approximately sinusoidal distribution. The lack of $\theta$-angles around 75° to 85° can be attributed to shot noise, resulting in non-valid intensity ratios. As discussed above, these ratios were fixed to the next possible ones, resulting in apparent large number of pixels of $\theta = 90^\circ$ and $\phi \sim 90^\circ$.

The markers shown in figure 5.15 define the position of the focus for recording stationary, individual time traces of PMI molecules which are shown in figure 5.17 and 5.18. The time traces were used to study the dynamics of these molecules as a function of time. Orientation traces were calculated from the recorded time traces, using the same correction factors as for the scanning images in figure 5.15 and applying a threshold criterion of 40 photons per bin. In order to avoid the rapid bleaching of the molecules, the laser power was decreased to 1 µW. The obtainable time resolution is mainly limited by the decreased count rate and was fixed to 5 ms for each time trace. The recording of the intensity time traces started shortly before opening the shutter for the laser light. For this reason, all measurements starting at time zero show an increase in intensity at the beginning.

Figure 5.17 1) shows a time trace where the mean intensity drops after 32 s by a factor of two. The common explanation of such a behaviour is that initially two molecules are within the excitation/detection focus, one of which bleaches after a certain time, whereas the other remains in an active state. Here, the calculated orientations are plotted as dots. The advantage
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Figure 5.17: part 1: Stationary time traces of two selected positions in figure 5.15. The black lines (right axes) represent the overall number of photons detected per 5 ms. The blue lines (blue dots in panel 1) show the calculated $\theta$-values, the red lines (red dots in panel 1) the $\phi$-values for each bin with valid intensity ratios. The green ellipse highlights a special feature.

of this method is that the density of dots represents some kind of histogram as a function of time. If the time range with one active molecule is compared with the time range with two active molecules, a superposition of two molecules is clearly visible for the orientation time traces. The bleached molecule had a smaller $\theta$-value and a larger $\phi$-value than the remaining one. Moreover, a very slow decrease in the overall intensity can be detected for the remaining molecule but no visible change in the orientation occurs. This can be attributed to either a movement of the laser focus away from the molecule or, but less likely, by a slow rotational motion of the molecule in the PMMA matrix.

Figure 5.17 2) shows a common feature of single fluorescent molecules namely blinking (for the blinking behavior of eGFP see, for example, references [39, 14], for PMI reference [37]). Fluorescent molecules can populate so called "dark-states", in which they are temporarily not fluorescent. The case shown here is unlikely in the way that the lifetime of the dark state is quite long (around 8 s). Often, as indicated by the green ellipse, the apparent lifetime of the dark state is much shorter. As expected, the calculated orientation remains unchanged even after the dark state. The short interruption of the dark-state (at around 66 s) is impossible to interpret. It could originate from a very short-lived fluorescent state or just from a fluctuation in the background intensity. Figure 5.18 3) shows a superposition of up to three molecules at the same place. The intensity time trace starts at a certain intensity level of around 350 photons per bin. After 1 s, the intensity increases to around 450 photons per bin and decreases suddenly to 200 photons per bin.
5.3. Experimental results

Figure 5.18: part 2: Stationary time traces of two selected positions according to figure 5.15. The black lines (right axes) represent the overall number of photons detected per 5 ms. The blue lines show the calculated $\theta$-values, the red lines the $\phi$-values for each bin with valid intensity ratios. The green dotted lines highlight special features.

直到所有分子都被漂白。将这种行为转化为一定数量的活性（荧光）分子，这将读作2-3-1。令人惊讶的一步是同时漂白两个分子。如图5.15所示，漂白的步骤导致$	heta$的对应值变化。如图5.4所示，检测到的强度是$	heta$的函数。事实上，$	heta$似乎随着强度的增加而减少。这表明分子正在改变其在PMMA基质中的取向。然而，快速跳回初始强度和取向再次令人惊讶，很难解释。这些例子清楚地表明，每一个单独的分子在其方向、闪烁行为、存活时间等特性上都有自己独特的特性。这些个体特征在整体测量中被平均化。

Polydiacetylene nanowires in porous alumina

聚丁二烯（PDA）是一种具有非常有趣特性的聚合物：它可以通过各种外部刺激引发突然的色彩转变，包括热退火，81
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$pH$ change, organic solvent exposure and mechanical stress [52, 9, 29]. PDA is obtained by photopolymerization or thermal polymerization of crystalline diacetylene and shows strong fluorescence [9]. The emission dipoles of the chromophores are uniformly oriented within crystalline grains. In addition, if molten diacetylene monomers were infiltrated into porous alumina templates, the confinement is expected to cause oriented crystallization of diacetylene, leading in turn to uniform crystal orientation in the polymerized PDA. Therefore, this model system is promising to demonstrate that the determination of the orientation of emission dipoles can be used to study the internal morphology of nanoobjects.

Molten diacetylene monomers were infiltrated into porous alumina templates with a pore diameter of around 400 nm, followed by photo-polymerization with UV light. After UV-exposure, the color of PDA turns from colorless to blue. Further heating above 80°C causes a color shift from blue to red. It was found that the red nanowires exhibit strong red fluorescence when excited with laser light with a wavelength of 544 nm.

How to make use of the proposed detection scheme for the emission dipole of single molecules in order to check the crystalline texture of the PDA inside porous alumina? As discussed above, the simultaneous detection of a large number of randomly oriented emitters will lead to $\theta \sim 60^\circ$ and $\phi \sim 45^\circ$ as long as the correction is done carefully. Even without correction, color-coded orientation images show uniformly colored areas, as long as the orientation of the emission dipoles is random (see figure 5.13 for comparison). The question arises if the infiltrated PDA contains randomly oriented emitters within the excitation/detection focus. For experiments with PDA-infiltrated porous alumina, the same experimental conditions were used as described in the previous subsection for the study of embedded PMI molecules. However, thanks to the bright fluorescence of PDA, the exciting laser intensity could be decreased to 8 nW. The aluminum substrate was removed from the porous alumina membrane by a selective wet-chemical etching step and the infiltrated alumina membrane was attached to the cover glass slide using immersion oil. Here, the requirement of a homogenous surrounding is not fulfilled if the focus is placed at the oil/membrane interface, because the refractive indices of both materials differ. Therefore, an adequate correction is almost impossible. Also, the interface might distort the emitted beam of light. For correcting the images, $\alpha_c$ was, more or less arbitrary, fixed to $\alpha_c = 48^\circ$, and the correction for the porization was chosen as $I_{90,c} = 1.3 \cdot I_{90,c}$. The threshold for calculating the orientations was set to 220 photons per pixel. Figure 5.19 a+b) shows the pseudo-color coded accumulated intensity of PDA-infiltrated porous alumina. The three detected intensities $I_{0,c}$, $I_{90,c}$ and $I_r$ were used to calculate $I_{sum}$. Whereas figure 5.19 a) represents a scanned area of 10 µm by 10 µm, the scanned area of figure 5.19 b) is 5 µm by 5 µm. Single infiltrated pores can be optically resolved using standard confocal optical microscopy. In figure 5.19 a), the hexagonal ordering of the infiltrated PDA indicating the positions of the pores can be seen quite well. Note that the pores are attached to the cover glass with the closed side of the membrane. Therefore, if the polymer does not perfectly infiltrate a single pore, the luminescence might be much weaker than in the case of a completely filled pore. The three intensity images belonging to figure 5.19 b) were used to calculate the orientation patterns of $\theta$ and $\phi$, as shown in figure 5.19 c+d). Surprisingly, the different areas corresponding to single pores do not have the same color, as one would expect for a large number of random emitters within the focus (see figure 5.13 for comparison). Nevertheless,
the calculated color range is not broad. To check the distribution of angles in more detail, each pattern was histogrammed pixel by pixel. The distributions of $\theta$-values and $\phi$-values are shown in figure 5.20 a+b). Both distributions do not follow the expected distributions of randomly oriented emitters for which one would expect a sinusoidal distribution of $\theta$-values and equal distributions of $\phi$-values. Especially the distribution of the $\phi$-values is much broader than in the case of an averaged emitter like the fluorescent latex bead in figure 5.14. However, the single spots corresponding to one PDA-nanowire are uniformly colored. Following the results, the polymerized PDA in a single pore is acting as a single emitter with a defined orientation of the emission dipoles and not as an averaged emitter consisting of a large number of randomly oriented emitters. Apparently, there are preferred orientations of the emitters in one pore. Additionally, it might be the case that this preferred orientation is a composition of a certain number of orientations. However, complete randomness would result in patterns much more uniform like those observed for fluorescent latex beads (figure 5.13). As a next step, it would be interesting to record intensity time traces of individual pores and to compare the histograms of the obtained orientations with the simulated ones.

It has to be considered that the infiltrated alumina membrane has a periodic structure close to
the wavelength of the emitted light, which might lead to further distortions. It might be helpful for future experiments to remove the cone-shaped pore bottoms of the alumina membrane and to record images in different depths of the PDA infiltrated membrane.

In conclusion, resolving the orientation of fluorescent polymers confined in nanoporous alumina offers a new possibility to determine textures of embedded fluorescent materials and probing ensembles of nanoobjects while resolving single nanoobjects simultaneously. This approach can also be applied to materials under shear stress or to drawn materials to probe their degree of anisotropy.

### 5.4 Discussion

The proposed method for orientation determination of the emission dipole of single fluorescent molecules, fluorescent latex beads or ensembles of fluorescent nanoobjects does not aim at determining the orientation with high accuracy. However, the main advantage is the obtainable time resolution. The presented scheme uses every single photon for the orientation determination, whereas the detection scheme proposed by Fourkas [26] rejects photons by using three different polarizations for detection. In comparison to CCD-chip based imaging techniques, as for example shown in reference [7], the proposed detection scheme has a number of advantages. For CCD-based imaging techniques the obtainable time resolution is limited by the frame rate of the camera (in the millisecond range). Furthermore, the photons from a molecule have to be distributed over a number of pixels reducing the signal-to-background ratio significantly. Finally, time-consuming fitting procedures have to be applied to the recorded images in order to obtain the orientation making a real-time determination impossible.

The method proposed in this work is straightforward. The time resolution is mainly limited by the countrate of the probe of interest. Using fluorescent latex beads, a time resolution of 500 µs was achieved. For single fluorescent molecules (PMI) the time resolution had to be decreased to 5 ms. Here, the countrate was decreased to about 100 photons per 5 ms by using a lower excitation intensity to prevent the rapid bleaching of the dye. The calculated orientation can be used to study the local viscosity around the fluorescent probe as shown in section 5.3.
In addition, the determination of oriented ensembles of emitters in crystals of fluorescent materials allows for analyzing the texture of these materials. Even if the relation of the transition dipole and the texture is not known so far, the proposed method can potentially be used as a complementary approach with X-ray diffraction methods and polarized infrared-spectroscopy to elucidate the supramolecular structure in the material under investigation. Moreover, the orientation distribution of the emission dipoles can be quasi simultaneously detected for a large number of fluorescent entities with high spatial resolution.