Tracking of single molecules as a powerful method to characterize diffusivity of organic species in mesoporous materials

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Abstract. The diffusion of individual fluorescent molecules can be observed by single-molecule tracking techniques and characterized by the analysis of their diffusional trajectory. Heterogeneities in the diffusivity that would pass undetected by conventional ensemble methods or fluorescence correlation spectroscopy are resolved by this method. This is demonstrated using four different examples in which we analyse the diffusion of single organic dye molecules in mesoporous materials. We show that this method can be used to obtain structural information from the inner structure of nanoporous materials with a resolution better than the optical diffraction limit.

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1. Introduction

Molecular diffusion in porous materials has been the subject of various studies, using methods as diverse as pulsed field gradient NMR [1], quasi-elastic neutron scattering [2], IR spectroscopy [3] and fluorescence recovery after photobleaching (FRAP) [4]. The range of techniques to investigate diffusion in these materials has been recently augmented by methods involving single-molecule spectroscopy (SMS) [5]–[9].

SMS is usually associated with (but not restricted to) the optical detection and spectroscopy of individual fluorescent molecules using different techniques of laser microscopy. The most important features of SMS and further references are provided in two recent reviews by Kulzer and Orrit [10] and by Moerner [11]. The diffusion of fluorescing dyes in a porous material can be investigated using two conceptually different SMS techniques.

The first method is the most direct way of investigating molecular diffusion. It is based on the analysis of a sequence of fluorescence microscopy images taken from very dilute samples. In such a sequence, or movie, the fluorescence originating from the individual dye molecules generates diffraction-limited patterns which may move from frame to frame and which can be tracked and subsequently analysed. The analysis of such trajectories is a method widely known as single-particle tracking, or SPT. A detailed description and further references can be found in the reviews by Saxton and Jacobson [12] and by Qian et al [13]. So far, SPT has mainly been applied to assess biophysical problems, for example to study the movement of dye-labelled lipids on lipid membranes [14] or to characterize the infection pathway of single viruses into a living cell [15]. The main advantage of this method is that it allows to characterize motion without the necessity of assuming a model. It shows directly the motion of the particles in space and time, revealing the eventual presence of different types of diffusional behaviour in the trajectories. A major limitation of this method is that it is sensitive only to slow processes: the acquisition time for one picture in a sequence has to be long enough to obtain unambiguous signals from the single molecule, usually it is in the order of 50 ms, but can be as short as 14 ms. This results in the observation of diffusion coefficients typically in the range of $10^{-8}$ to $10^{-12}$ cm$^2$ s$^{-1}$.

The second method is based on the observation of individual molecules diffusing in and out of a well-defined focal volume, or detection volume, of a microscopy apparatus. It gives a statistical analysis of the fluctuations of the detected fluorescence intensity in time $I(t)$, which has fluorescence bursts of a characteristic duration. The method is well known as fluorescence correlation spectroscopy (FCS) [16, 17]. This approach allows us to determine a wide range of diffusion coefficients, from $10^{-8}$ to $10^{-4}$ cm$^2$ s$^{-1}$. One limitation of this method is that the autocorrelation function of the intensity $g^{(2)}(\tau) = \langle I(t)I(t+\tau)\rangle/\langle I(t)\rangle^2$ has to be matched to a hypothetical expression derived from a model of the diffusion process in order to obtain a parameter like the diffusion coefficient. Another serious limitation of FCS in the characterization of diffusion is that the method loses the spatial and temporal information contained in the trajectory of an individual molecule.

In this paper, we present four different investigations of single molecules diffusing in mesoporous materials that illustrate the capabilities of the single molecule tracking method. We will concentrate on the potential of this relatively new and versatile technique taking several examples from different so-called host–guest systems rather than elaborating specifically on one of these systems in detail.

Firstly, we show how this technique reveals the presence of distinct diffusional behaviour on going from molecule to molecule. Secondly, we observe a spatial heterogeneous behaviour
in the trajectory of one individual molecule, which is trapped in a small confinement and is then released. In the third example, we observe a deviation in the distribution of step sizes from the distribution expected for normal diffusion, indicating an anomalous movement on the relevant time scale. This anomaly could be due to molecular-size barriers and adsorption sites present in the material. In the last example, we observe and analyse trajectories of molecules diffusing in a structured material. The diffusion in this case appears to be normal, but spatially anisotropic, possibly reflecting the structure of the material. This anisotropy becomes apparent in the distribution of the angles between successive steps, with few significant steps perpendicular to the overall trajectory.

2. Experiment, results and discussion

Images, and sequences of images, were recorded with a wide-field imaging setup, schematically depicted in figure 1(a). Two fluorescent dyes, a derivative of terrylene diimide (TDI) and a streptocyanine dye (9A1), were used in these studies. Their chemical structures are depicted in figure 1(b). Both dyes have their emission maxima at a wavelength of about 660 nm, and can be excited with an He–Ne laser (NEC) at 633 nm. The laser beam used for the excitation of the molecules is expanded and focused onto the back-focal plane of a microscope objective (Nikon Plan Apo 100×/1.4 oil; the microscope stand is a Nikon eclipse TE200). The dyes in the

Figure 1. (a) Schematic drawing of the wide-field imaging setup. (b) Chemical structures of the used derivative of TDI and streptocyanine dye (9A1).
illustrated area of the sample are excited and fluoresce. This fluorescence is collected by the same objective, separated from backscattered laser light with a combination of filters (dichroic mirror 640 nm cutoff and band-pass BP730/140 AHF) and imaged onto a CCD detector. The lateral resolution of this setup is almost at the diffraction limit or about 300 nm. The detection (or focal) depth resolution of this setup is about 3 μm. In other words, the acquired images are the two-dimensional projection of a 3 μm-thick slab.

Two different detector systems were used in this work: (a) a back-illuminated EMCCD (Andor, iXon DV897, 512 × 512 frame transfer CCD) produces images with 118 nm per pixel, and at a frame rate of 70 s⁻¹ (14.3 ms per frame, including read-out delay of 1.8 ms). This setup was used to acquire data for the first example (cf figure 3) discussed in section 2.1. (b) An intensified MCP-CCD camera (PentaMAX-512EFT, 512 × 512 pixel, Princeton Instruments) renders images with 169 nm per pixel, and at a frame rate typically between 10 and 25 s⁻¹ (or between 100 and 40 ms per frame, including read-out delay of <1 ms). This setup was used for all the other examples described in sections 2.2–2.4. The size of the images and the length of the image sequences can be adjusted from measurement to measurement (by setting the region of interest (ROI) on the CCD chip). In the measurements with detector (a), sequences of 500 frames were acquired with an ROI of 250 × 200 pixels (about 30 × 24 μm). Sequences recorded with detector (b) consist of 1000 frames and an ROI varying around 150 × 150 pixel (about 25 × 25 μm).

In the sequence of images, the molecules appear as diffraction limited bright spots, as shown in figure 2. As mentioned before, these spots may move from frame to frame, as the molecules diffuse in the sample. The positions of these spots are obtained by analysing the films and tracking the individual patterns using computer software. In essence, the programme obtains the positions and the positioning errors by fitting a two-dimensional Gaussian to the individual patterns in every frame \( n \):

\[
I(x, y) = A_n \exp\left\{-(x - x_n)^2 + (y - y_n)^2\right\}/2\sigma_n^2\right\}.
\]

In this equation, \( A_n \) is the amplitude of the Gaussian (it is given as a greyscale value in the images). Parameter \( \sigma_n \) is related to the full width at half maximum of the pattern by
FWHM = \(2(2 \ln 2)^{1/2}\sigma_n\). The parameters \(x_n\) and \(y_n\) give the central positions of the fitted pattern. The errors in the four parameters \(A_n\), \(x_n\), \(y_n\) and \(\sigma_n\) vary for each analysed spot because the fitting accuracy depends on the signal-to-noise ratio [20]. The errors in \(x_n\) and \(y_n\) represent the positioning accuracy and are typically well below the (lateral) optical resolution limit of the microscopy apparatus (i.e. 15–50 nm in comparison with about 300 nm optical resolution). A more detailed description of the tracking procedure is given in [9]. The output of this software consists of a sequence of positions for each tracked molecule, which result in the trajectories of the individual molecules. Before presenting the individual observations, we should recall that the experiment samples continuous motion at discrete times. These discrete times are significantly above the individual molecular collision times. Even so, we term the change in position between frames as ‘step’ and show the trajectories connected by broken lines.

We would also like to provide a final note on the appearance and disappearance of single-molecule patterns in the images. There are two main reasons why the patterns generated by the individual molecules appear and disappear from the image sequence at different times: firstly, the molecules can enter and exit the detection volume (the focal slab) due to diffusion perpendicular to the observation plane and, secondly, the molecules might cease to fluoresce due to an irreversible photo-induced reaction (called photobleaching). In the case of spin-coated samples (described in sections 2.1, 2.3 and 2.4), which have a thickness of 100–200 nm, the diffusion into and out of the detection volume is suppressed, thus the main reason for the disappearance of the molecules in these cases is photobleaching. In the case of more voluminous samples (described in section 2.2), the detection volume is placed entirely within the material. The diffusion perpendicular to the focal plane is perceived as a blurring of the patterns, and is the predominant reason for molecules appearing and disappearing in the sequences.

2.1. Distinct diffusional behaviour on going from molecule to molecule

In the first example, we observe the diffusion of TDI molecules incorporated into a porous, spin-coated SBA-16 silica structure. The pores of this material consist of interconnected spherical voids arranged in a body-centred-cubic structure. The exact size and shape of the spherical voids vary. They have a diameter of approximately 10 nm. The size, shape and geometrical arrangement of the pores are evaluated from transmission electron micrographs, x-ray diffractograms and adsorption isotherms. The individual TDI molecules can be observed as single diffusing molecules via their fluorescence as described in the previous section (the movie is available in the supportive material: TDI_SBA16_full; TDI_SBA16_cropped). In figure 3 the diffusion of two molecules in the same sample are described by their trajectories and the respective analysis. A significant difference in diffusivity between the two molecules is readily observed. In figure 3(a), the trajectory of molecule A covers a large region in comparison to molecule B, which is found localized in a significantly smaller region. The diffusion of molecule A can be compared with a two-dimensional random walk, in which the mean-square displacement (MSD), \(\langle r^2(t) \rangle\), obeys

\[
\langle r^2(t) \rangle = 4Dt.
\]  

The MSDs for every time lag \(t = n\delta t\) (\(n\) is the number of frames between two positions and \(\delta t\) the acquisition time; \(\delta t = 14\) ms in this specific example) are obtained directly from the trajectory.

This sample was prepared and characterized by the group of Professor Dr Thomas Bein.
Figure 3. Analysis of two trajectories of individual TDI molecules diffusing in SBA-16 with a nominal pore diameter of 10 nm. The time lag is 14 ms between frames. (a) The trajectories show a pronounced difference. The trajectory of molecule A contains 223 positions and shows visible mobility. The trajectory of molecule B consists of 294 steps and remains confined to a small area. (b) The MSDs calculated for all steps of the two trajectories are plotted versus time. The effective diffusion coefficient evaluated for molecule A is $D_E = 1.7 \times 10^{-9}$ cm$^2$ s$^{-1}$ under the assumption of random motion. Molecule B shows no detectable movement ($D_E < 10^{-13}$ cm$^2$ s$^{-1}$). (c) The angular distribution for two consecutive steps for the two molecules also shows a difference. No preferential step angle is found in trajectory A. The significant number of backward steps ($\pm 180^\circ$) in trajectory B is probably due to an artifact (cf text). The straight line in both graphs corresponds to a value of 1/36 expected for an equal distribution of angles. (d) The step-size distribution shows a maximum at a step length of 72 nm for the mobile molecule A. The maximum in the step-size distribution of molecule B is of the same order as the positioning accuracy, which is 15 nm.

assuming that any starting point in the trajectory is equally valid. A linear regression through the data for this trajectory gives an effective diffusion coefficient of $D_E = 1.7 \times 10^{-9}$ cm$^2$ s$^{-1}$ (see figure 3(b)).

In contrast to this, molecule B is found to be immobile within our positioning accuracy (of approx. 15 nm for this molecule). In this specific case, it is not possible to infer whether the
molecule is trapped within a void of <15 nm or if it is adsorbed on a molecular site (i.e. localized to within a few Å). This could be due to functional groups on the walls of the pores interacting with the dye molecule [7]. The effective diffusion coefficient of molecule B is \( D_E < 10^{-13} \text{ cm}^2 \text{ s}^{-1} \) (cf figure 3(b)). Molecules localized on a scale of length smaller than the positioning accuracy show an apparent jittering movement. This jittering is an artifact and results from the errors when the position is extracted from every frame. This artificial jittering becomes apparent in the distribution of angles between successive steps, shown in figure 3(c) for molecule B. The angles between successive steps are defined as follows: forward movement corresponds to 0° and backward movement to ±180°. Steps on the left correspond to −90° and those on the right correspond to +90°. In this histogram, it is possible to see that backward steps are over-represented for molecule B. The mobile molecule A does not show any preferential angle, confirming the randomness of the trajectory.

The distribution of step sizes for both molecules is shown as a histogram in figure 3(d). This distribution corresponds to the distribution expected from the normal diffusion theory [18]:

\[
q(r, t) = \frac{2r}{\langle r^2(t) \rangle} \exp \left( -\frac{r^2}{\langle r^2(t) \rangle} \right),
\]

In this case, \( t = 14 \text{ ms} \). The maxima of the distributions are found at 72 nm for molecule A and at 15 nm for molecule B (15 nm corresponds to the average positioning accuracy for this molecule).

The behaviour of the two molecules described above represents two extreme examples: one diffusing and one confined, coexisting in the same sample. Measurements of many molecules in such a sample allow to obtain the distribution function of the diffusional behaviour. The width of such a distribution can vary strongly and even subpopulations can be observed. In this specific case, the two molecules belong to two distinct sub-populations i.e. an immobile population and a diffusing population with a broad distribution of diffusion coefficients around \( 10^{-9} \text{ cm}^2 \text{ s}^{-1} \). Other methods that probe an ensemble of molecules do not readily resolve these sub-populations, FCS in particular is not sensitive towards sub-populations of immobile molecules. The absolute confinement of the immobile molecule in this example is indicated by the observation that (of about 100 investigated trajectories in this sample) no intermediate case, i.e. a molecule becoming trapped or released from a trap is observed. The molecules in this sample can be considered to be either mobile or immobile. (We note that the dye molecules are added during the synthesis of the material and could therefore be completely enclosed by the solid matrix.)

2.2. Spatially heterogeneous behaviour in the trajectory of individual molecules: traps

Whereas in the first example the heterogeneity was shown between two differently behaving molecules, in the second example we find heterogeneity within the trajectory of one individual molecule. A detailed analysis of the diffusion in this host–guest system has been given in [9]. In this material, streptocyanine 9A1 molecules are incorporated into the pores of a sol–gel glass with a nominal mesopore diameter of 3 nm. Such sol–gel glasses are non-ordered materials. Therefore, the sizes and shapes of the pores in this material are more heterogeneous than the ones in the first example. In figure 4(a), the trajectory of a single 9A1 molecule shows a visible trapping region (the movie is provided in the supportive material: VG5_M3_cropped). The ordinary analysis of the MSD versus time averages over all steps of the trajectory, which gives an effective diffusion coefficient of \( D_E = 1.5 \times 10^{-10} \text{ cm}^2 \text{ s}^{-1} \), under the assumption that all positions in this trajectory
Figure 4. Analysis of a trajectory of an individual 9A1 molecule diffusing in a sol–gel–glass with mesopore diameter of 3 nm. A total of 40 positions with a time lag of 100 ms are observed. (a) The molecule starts in what appears to be a trap of about 25 steps and is released. (b) The effective diffusion coefficient for the entire trajectory ($D_E$) is found to be $D_E = 1.5 \times 10^{-10} \text{cm}^2 \text{s}^{-1}$. A separation of the trajectory into a fast and a slow population gives $D_M = 5.8 \times 10^{-10} \text{cm}^2 \text{s}^{-1}$ for the fast population and $D_I < 10^{-11} \text{cm}^2 \text{s}^{-1}$ for the slow population. (c) The separation of the two populations is obtained by a statistical evaluation of squared step lengths (for details see the text). Here, the relative rank is plotted for three time lags (0.1, 0.2 and 0.3 s), clearly showing the presence of a slow and a fast population. The respective values for the MSD are obtained by fitting this graph to equation (7). (d) The step length histogram for these three steps has a constant maximum for the immobile population at 35 nm, which is comparable to the positioning accuracy. The maxima for the mobile population are at 195, 218 and 245 nm for 0.1, 0.2 and 0.3 s.

are equally valid starting points for the analysis of the diffusion. In this particular example, it is apparent that this assumption does not hold. It would be possible to treat the ‘trapped’ region and the ‘diffusing’ region separately; however this would imply an arbitrary ‘cut’ of the trajectory. Trajectories with less visible trapping regions would be difficult to ‘cut’ appropriately. The MSDs $\langle r^2(t) \rangle$ for every time lag $t = n \delta t$ (the acquisition time $\delta t$ is 100 ms in this specific example) can be obtained in a different way, namely by analysing the distribution of squared displacements $u$.
(with \( u = r^2 \)), which is given by

\[
p(u, t) = \frac{1}{\langle r^2(t) \rangle} \exp \left[ -\frac{u}{\langle r^2(t) \rangle} \right]. \tag{4}\]

The cumulative probability distribution is thus

\[
P(U, t) = \int_0^U p(u, t) \, du = 1 - \exp \left[ -\frac{U}{\langle r^2(t) \rangle} \right] \tag{5}\]

and gives the probability that \( u \) does not exceed a value \( U \). The complementary function

\[
C(U, t) = 1 - P(U, t) \tag{6}\]

is a simple decaying exponential function with decay constant \( \langle r^2(t) \rangle \). The experimental values of the individual squared displacements \( u \) obtained for a trajectory at a specific time lag can be sorted (longest squared step first) and assigned a rank \( j \). In a total of \( N \) steps, the relative rank \( j/N \) corresponds to \( C(U, t) \). In figure 4(c), the relative rank \( j/N \) of every step is plotted against the squared step length for three different time lags 0.1, 0.2 and 0.3 s (in this case \( N = 39, 38 \) and 37 respectively). The presence of two populations in the semi-log plot is well visible in this case. The data are fitted to a bi-exponential decay corresponding to equation (6), yielding two decay constants \( \langle r^2(t) \rangle_M \) and \( \langle r^2(t) \rangle_I \). The obtained MSDs are plotted versus time in figure 4(b). A linear fit through the two sets of data show that the ‘mobile’ part of the trajectory is comparable to normal diffusion (equation (3)) with a diffusion coefficient of \( D_M = 5.8 \times 10^{-10} \text{ cm}^2 \text{ s}^{-1} \). The ‘immobile’ part has a diffusion coefficient \( <10^{-11} \text{ cm}^2 \text{ s}^{-1} \), which would be the detectable minimum for this amount of data. In cases where more data points are available, even smaller diffusion coefficients can be determined. The step-size histograms depicted in figure 4(d) confirm the presence of two distinct sub-populations of steps in this trajectory. For similar reasons as stated above, we cannot differentiate whether a molecule is trapped in a geometrical trap with an effective size below 35 nm (the mean positioning accuracy in this trajectory) or whether the molecule is immobile on a molecular scale of length. Further evidence for adsorption at molecular sites would be obtained by measuring the rotational diffusion of such single molecules [19].

2.3. Heterogeneity in the distribution of step sizes: two sub-populations

The presence of diffusional traps, as described in the previous example, must not necessarily be localized to a specific point of the trajectory or of the sample. In this third example, we observe and analyse a TDI molecule diffusing in a spin-coated SBA-15 material (the movie is provided in the supplementary material: TDI_SBA15_full). This material has interconnected cylindrical pores of 6 nm width in average.\(^3\) A trajectory consisting of 482 individual steps is observed with a time lag of \( \delta t = 43 \text{ ms} \). The trajectory given in figure 5(a) does not show any special structure or feature. The analysis of MSD given in figure 5(b) indicates that the overall motion can be compared with normal Brownian motion, with an effective diffusion coefficient \( D_E = 5.6 \times 10^{-10} \text{ cm}^2 \text{ s}^{-1} \). We recall that the values for the MSD for a time lag \( t \) are the average over all steps separated by this specific lag—the information contained in each step is discarded

\(^3\) See footnote 2.
Figure 5. Analysis of a trajectory of a TDI molecule diffusing in SBA-15. The time lag is 43 ms between frames. (a) The trajectory consists of 482 steps. (b) The MSDs calculated for all steps are plotted versus time. The overall diffusion is comparable to a random walk with a diffusion coefficient of $D_E = 5.6 \times 10^{-10} \text{ cm}^2 \text{ s}^{-1}$ (thin line). (c) The ranked step length distribution deviates from a unimodal distribution (cf thin line in the residue plot represents the deviation of the experimental data from the best fit). A better fit is obtained by assuming a bimodal distribution (cf white and thick lines in the residue plot). (d) This bimodal distribution is best visualized in the step length distribution histogram. Two step types occur in the trajectory: large steps (maximum at 174 nm) and short steps (maximum at 83 nm). It is not possible, in this trajectory, to spatially separate fast and slow regions.

by averaging. An analysis of the distribution of step sizes should confirm the presence of one or more populations, because the information contained in each step is retained. As in the previous example, the relative rank $j/N$ corresponds to the complementary function of the cumulative probability distribution ($j/N$ is obtained by sorting the individual steps and assigning each step a rank). In a random walk, the distribution of displacements (or step lengths) $r$ in time $t$ is given by equation (3). The cumulative probability distribution is thus

$$P(r, t) = \int_0^R q(r, t) \, dr = 1 - \exp \left[ -\frac{R^2}{\langle r^2(t) \rangle} \right].$$

(7)
$P(R, t)$ gives the probability to find a step with a length between 0 and $R$. The complementary function

$$C(R, t) = 1 - P(R, t)$$

in the case of normal diffusion is a Gaussian centred at the origin. The relative ranks are plotted versus displacement in figure 5(c). A fit to a single Gaussian shows a significant deviation from the simple model (cf thin line in the residue plot). When an additional component (a second Gaussian) is added to the model, the analysis yields an acceptable fit to the data (cf thick line in residue plot). Two populations with maxima at a step size of 83 and 174 nm are found. A more intuitive visualization of this distribution is given in figure 5(d) where the histogram of step sizes is shown (note that information is lost by binning the data). The straight curve in the graph is obtained from equation (3). In principle, one could expect that the sample shows regions where a molecule can move fast and other regions where it predominantly moves slowly. However, in the case presented above, the analysis shows that long and short steps are distributed equally throughout the entire trajectory, rather than being localized at specific regions. Even though we can identify two sub-populations in the step sizes, these populations do not appear spatially separated.

### 2.4. Trajectories of molecules diffusing in a structured material

In figure 6(a), another type of trajectory of a TDI molecule diffusing in SBA-15 is shown. The molecule can be followed for 103 frames, and moves on an S-shaped trajectory (movies are in the supplementary material: TDI_SBA15struc_fr45_169; TDI_SBA15struc_full). Particularly intriguing is the fact that it follows the same trajectory back and disappears close to the starting point (on the upper right). No analysis based on step lengths will reveal any deviation from a two-dimensional random walk. An MSD versus time plot (cf figure 6(b) yields an effective diffusion coefficient of $D_E = 2.1 \times 10^{-9}$ cm$^2$ s$^{-1}$. The step-size distribution shown in figure 6(c) shows a maximum at a step length of about 145 nm (with an error of ±20 nm, due to the limited statistics). A possibility to reveal a deviation from an isotropic random walk in two dimensions is given by the analysis of angles between steps. In this particular case, a significant population is found at 0° and ±180°, whereas significantly few steps have an angle of ±90°. This means that this trajectory bears more similarity to a one-dimensional random walk than to a two-dimensional one. If that is the case, the trajectory reflects a real structural feature within the host material (an S-shaped channel, for example). For this reason, it would be more appropriate to assume a diffusion along a tortuous pore instead of a two-dimensional plane. Such an analysis is, however, difficult to carry out with trajectories of molecules diffusing in a material from which the exact shape and interconnectivity of the pores is not known.

### 3. Summary

The four examples presented show how intricate the diffusional behaviour in an apparently simple sample (organic dye molecule in a mesoporous material) can be and how this is resolved in a straightforward way by observing and analysing the movement of individual molecules. In the analysis of diffusion, all parameters may point to a heterogeneous behaviour. In the first case, we...
Figure 6. Analysis of the trajectory of a TDI molecule diffusing in SBA-15. The time lag is 70 ms between frames. (a) The trajectory consists of 103 steps and appears to be structured. (b) The MSDs calculated for all steps are plotted versus time. An effective diffusion coefficient of $D_E = 2.1 \times 10^{-9} \text{ cm}^2 \text{s}^{-1}$ is found for this trajectory assuming a two-dimensional random walk. (c) The step-length distribution for the trajectory is depicted as a histogram. The distribution has a maximum at 143 nm. (d) The distribution of the angles between successive steps for the trajectory indicates that there is a preferential movement forward and backward and that there is less movement perpendicular to the trajectory. The straight line corresponds to the value of $1/36$ and corresponds to the expected distribution for an isotropic random walk in two dimensions.

see direct proof of the somewhat self-evident conjecture that different single molecules behave differently. The chosen example is an extreme one, showing a virtually immobile molecule and a diffusing molecule coexisting in the same sample. However obvious the observation, we do not know of any other method that easily resolves the coexistence of mobile and immobile molecules in distinct spatial regions of such a sample.

In the second example, a molecule appears to get out of a trap. In this case, it can be shown that a trajectory is not characterized sufficiently by just checking the linearity of a mean square displacement versus time plot. The chosen trajectory represents a particularly clear-cut case.

The third case can be considered a special case of the second. A molecule is found to move with two distributions of step sizes. Besides that observation, all other diffusion properties point
to a normal isotropic diffusion in two dimensions. In this trajectory, it is not possible to find specific regions with slow or fast diffusion. The molecule obviously experiences some form of hindrance in its diffusion. One possibility to explain this kind of movement would be that the molecule is affected by adsorption sites or geometrical barriers distributed equally on a spatial and temporal scale just below the one resolved by the microscopy apparatus. A hindrance taking place on a much shorter temporal or spatial scale would simply get averaged out, resulting in an effectively reduced diffusion coefficient.

Methods that probe ensembles of molecules may detect sub-populations of slow and fast moving molecules, however one cannot directly ascertain if this population is separated from molecule to molecule (as in the first example, assuming that the immobile molecule in question is truly confined) or if one and the same molecule shows this heterogeneous behaviour (as in the second and third examples). This can be shown most easily and directly by evaluating the trajectories of individual molecules.

In the fourth example, we observe a molecule diffusing back and forth on what appears to be an S-shaped trajectory. It is only the analysis of angles between steps that points towards a movement that is more similar to a one-dimensional diffusion than to a two-dimensional random walk. In this case, the trajectory reflects some form of structure (e.g. a tortuous channel) in the material. We emphasize that this structural information is obtained on a large area of the sample (25 µm × 25 µm) and with a comparatively high spatial resolution of about 50 nm (conventional optical methods do not provide such a high spatial resolution). Furthermore, the information is obtained from the volume of a sample in a non-destructive way. The resolution of electron microscopy images (particularly TEM) is significantly higher. However only a comparatively small region, about 100 times smaller than in our case, can be observed. Besides, TEM is a destructive method and requires a more complicated sample preparation. Nevertheless, the combination of the optical single-molecule tracking with TEM raises the possibility of obtaining a more complete picture of the diffusion processes taking place in these materials. TEM images would reveal to what extent the pores are interconnected or if the tortuosity of the channels is comparable to the shape of the observed trajectories. The structured trajectories obtained by single-molecule tracking are particularly intriguing and are currently being subjected to more thorough scrutiny.

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