Microsecond Single-Molecule Tracking (μsSMT)

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ABSTRACT Here we report on a method to track individual molecules on nanometer length and microsecond timescales using an optical microscope. Our method is based on double labeling of a molecule with two spatially distinct fluorophores and illuminating it with laser pulses of different wavelengths that partially overlap temporally. We demonstrate our method by using it to resolve the motion of short DNA oligomers in solution down to a timescale of 100 μs.

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A persistent challenge in biophysics is measuring the motion of single biomolecules on short length scales and timescales (1). Recent advances in superresolution techniques (2–5) have pushed the resolution limit of optical microscopes down to the nanometer range. However, these techniques are severely limited in their temporal resolution because they either require scanning the sample (as in stimulated emission depletion microscopy) or acquiring stochastically occurring signals (as in stochastic reconstruction microscopy (STORM)) and photoactivation localization microscopy (2). Hence, they cannot access the fast dynamic interaction of mobile biomolecules with nanometer-sized structures. For example, the interaction of a lipid molecule diffusing in a cell membrane (diffusion coefficient $D \approx 1 \, \mu m^2/s$) with a membrane substructure that has a characteristic length $L$ of ~50 nm occurs on a timescale $t = L^2/4D \approx 1 \, ms$. Consequently, observing the interaction of molecules with nanometer-sized structures requires techniques with submillisecond temporal resolution.

Here we present what is, to our knowledge, a new camera-based, thus highly parallel, single molecule tracking (SMT) method that fulfills this requirement. The two major factors which determine the temporal resolution of camera-based SMT (6) are the acquisition speed of the charge-coupled device (CCD) camera and the illumination time necessary to detect the optical probe. During the illumination time, the probe must emit enough photons to be detected above the read-out noise of the camera. At the same time, the read-out noise generally increases with the read-out speed of the camera. Consequently, SMT on microsecond timescales could so far only be realized with colloidal gold probes (7) or highly elaborate optical detection schemes (8–10). Although colloidal probes are bright and optically stable, they are not optimal for biological applications because of their large size and unspecific binding issues. Existing fast detection schemes for single molecules can only track one molecule at a time (8–10) or have a small field of view (10).

In contrast, our method uses small fluorophores in combination with a simple detection scheme—a slow-scan, low readout-noise CCD camera system—to achieve microsecond single-molecule tracking (μsSMT). μsSMT is comparable to STORM in that it uses two fluorescent probes on a single molecule. In contrast to STORM, these probes are placed so far apart that no energy transfer can take place between them and thus the two are excited separately.

The emitted signal of the two fluorophores is collected onto separate regions of a CCD chip with the help of a dichroic wedge (see Fig. S1 in the Supporting Material). In this way, the two fluorophores are separately excited and their fluorescent signals recorded independently of each other. Therefore, we can resolve the position of a single molecule with a nanometer resolution at two different points in time.

To demonstrate our method, we used short DNA-oligonucleotides in solution that were labeled with two fluorophores, Cy3B and ATTO647N. These probes were then separately illuminated by two lasers at wavelengths of 514 nm and 639 nm, respectively, during a certain illumination time $t_{ill}$. The pulses of the two lasers were temporally offset by a small time lag $\Delta t$ (see Fig. 1 a). In principle, we can make $\Delta t$ arbitrarily small. The actual temporal resolution achievable in practice is primarily determined by the molecule’s diffusion coefficient, the accuracy with which we can determine the molecular positions, and the total number of single-molecule positions measured.

Because the illumination time was finite, we did not measure the actual positions $\mathbf{r}(t)$ and $\mathbf{r}(t + \Delta t)$ of the molecule. Instead, our method measured the positions

$$\overline{\mathbf{r}(t)}$$ and $\overline{\mathbf{r}(t + \Delta t)}$

averaged over the illumination time $t_{ill}$. Due to this temporal averaging, the mean squared-displacement (MSD) calculated from measured positions was different from the actual MSD of the molecule (11). For ergodic and stationary processes there is, however, a simple relation between the observed (averaged) MSD($\Delta t$) and the actual MSD of the molecule.
FIGURE 1 Illumination scheme and position determination. (a) Laser pulses of duration $t_{ill}$ are temporally offset by a time lag $\Delta t$. (b) Raw image from the Cy3B channel. (c) Raw image from the ATTO647N channel. \( \Delta t = 0 \) ms. Scale bar is 5 \( \mu \)m. (d) Correction for chromatic aberration. The scheme shows positions in the Cy3B (green dots) and ATTO647N (red dots) channel which correspond to the same physical positions. (e) Identified single-molecule signals from the images shown in panels b and c, corrected for chromatic aberration.

\[
\text{MSD}(\Delta t) = \left\langle (\mathbf{r}(t + \Delta t) - \mathbf{r}(t))^2 \right\rangle
\]

\[
= \frac{1}{t_{ill}^2} \int_{-t_{ill}}^{0} dt' \int_{-t_{ill}}^{-t_{ill} - \Delta t} dz (\text{MSD}(\Delta t + \epsilon) - \text{MSD}(\epsilon)).
\]

(1)

Because the microscope projects the three-dimensional movement of the molecules in solution onto a two-dimensional plane,

\[
\text{MSD}(\Delta t) = 4D \Delta t
\]

For regular diffusion, Eq. 1 was evaluated analytically (see the Supporting Material for the derivation of the general relation and the analytical evaluation).

\[
\text{MSD}(\Delta t) = 4D \times \begin{cases} \frac{\Delta t^2}{t_{ill}} - \frac{1}{3} \frac{\Delta t^3}{t_{ill}^2} & \text{for } \Delta t \leq t_{ill} \\ \Delta t - \frac{1}{3} \Delta t^3/t_{ill} & \text{for } \Delta t \geq t_{ill} \end{cases}
\]

(2)

Below we will show that the measured MSD(\(\Delta t\)) indeed behaved as predicted by Eq. 2.

The calculation of a molecule’s MSD requires the determination of its position. In our experiments, we chose a low density of molecules such that molecules were resolved individually and their position determined with an positional accuracy (6). Fig. 1 illustrates the process of position determination. Fig. 1, b and c, shows typical raw signals from several molecules dual-labeled with Cy3B (Fig. 1 b) and ATTO647N (Fig. 1 c). The positions of the fluorophores were determined by fitting two-dimensional Gaussians approximating the point-spread function of the microscope. The positional accuracy achieved was between 40 and 50 nm (see the Supporting Material). Because the signals from the two fluorophores labeling the single molecule had to be correlated, eliminating chromatic aberration was important. We established the correspondence between the two signals by using fluorescent beads which were observable in both channels, Fig. 1 d shows the corrective shifts which were applied to the signals in the green (or red) channel, respectively. Fig. 1 e shows the single-molecule positions determined from Fig. 1, b and c, and corrected for chromatic aberration. Note that due to the finite labeling efficiency and bleaching, some molecules carried no or only one of the labels.

To determine the MSD of the molecules, we correlated the positions that were measured in the two detection channels by particle image cross-correlation spectroscopy (PICCS, see the Supporting Material), an extension of particle image correlation spectroscopy (PICS) (12). Because PICCS relies on highly accurate detection of single-molecule positions, our method is not diffusion-limited and unaffected by autofluorescence and noise, both of which are common problems faced in the conventional imaging methods. Fig. 2 shows a comparison of MSDs obtained experimentally under different solvent and illumination conditions. Equation 2 fits the experimental data in all cases. The inset table in Fig. 2 b compares the diffusion coefficients determined from the fit. As to be expected, the diffusion coefficient decreased with increasing amount of dextran T5000 corresponding to an increased viscosity.

Compared to the situation without the dextran, the diffusion coefficient decreased by a factor of 3.1 ± 0.9 in the solution with 5% dextran T5000 and by a factor of 5.8 ± 1.0 in the solution with 10% dextran T5000 (see inset table in Fig. 2 b). We confirmed our results by independent fluorescence correlation spectroscopy measurements (see the Supporting Material) of the same DNA construct in the same solvents in which we found factors of 3.20 ± 0.04 and 6.05 ± 0.06 between the corresponding diffusion times, respectively. Interestingly, the diffusion coefficient did not scale with the bulk kinematic viscosity which increased by a factor of 6.2 ± 0.1 in the solution with 5% dextran T5000 and by a factor of 20.9 ± 0.2 in the solution with 10% dextran T5000, as determined by bulk viscosity measurements. This deviation probably reflects the fact that solvent and solute molecules were comparable in size for which the Stokes-Einstein relation is invalid.

For nonoverlapping laser pulses (\(\Delta t > t_{ill}\)), the observed MSD was shifted down by \((4/3) D_{ill}\) (see Fig. 2 d), in agreement with earlier results (11). For overlapping laser pulses (\(\Delta t < t_{ill}\)), the MSD was clearly nonlinear and approached...
Finally, μsSMT can be easily adapted for measurements in cells. For example, a membrane receptor could be labeled with two genetically encoded tags, one at a cytosolic domain, and one extracellular. The separation of the two labels by the cell membrane would prevent energy transfer between the fluorophores. Because only low laser intensities are required, the method is suitable for live cell recordings. Consequently, our method will likely aid in providing what we believe will be new insights into the dynamic behavior of molecules in cells on very short length- and timescales.

SUPPORTING MATERIAL

Supporting methods and theoretical derivations are available at http://www.biophys.j.org/biophys/supplemental/S0006-3495(10)05294-X.

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REFERENCES and FOOTNOTES


Microsecond Single-Molecule Tracking (μsSMT)
Supplementary Material

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1 DNA construct

The molecule tracked in the experiments described here was a fluorescently labeled 155
basepair (bp) DNA construct containing a 601 nucleosome positioning sequence [1].
The DNA was prepared by PCR and was labeled with biotin, Cy3B and ATTO647N
by incorporation of fluorescently labeled, HPLC purified primers (IBA GmbH). PCR
primers were as follows: 5’-TTGG CTGGAGAATC CCGGTGCGA GGGCGCTCAAA
TTGGTCGTAG ACAGCTCTAG CACCGCTTAA ACCCACGTCG GCGCTG-3’
(Cy3B-labeled nucleotide is underlined) 5’-biotin-TTGGACAGGA TGATATATGC
TGACACGTGC CTGGAGACTA GGGAGGATTCTC GCCTGCGGG GTAAAAAGCGC
GGGGACAGC-3’ (ATTO647N-labeled nucleotide is underlined) In the DNA the
Cy3B and the ATTO647N were located 76 bp (24 nm) apart. Since this distance was
significantly bigger than the Förster radius of the fluorophores (≈ 5.5nm) there was
no FRET as confirmed by FCS experiments (data not shown).

2 Single-molecule microscopy

The experimental setup for single-molecule imaging has been described in detail pre-
viously [2]. Briefly, the microscope (Axiocam 100; Zeiss, Oberkochen Germany) was
equipped with a 100x oil-immersion objective (NA=1.4, Zeiss, Oberkochen, Ger-
many). The samples were illuminated for \( t_{ill} = 1\, ms \), 3\, ms or 5\, ms by an Ar\(^+\) laser
(Spectra Physics, Mountain View, CA, USA) with a wavelength of 514\, nm and a
30mW diode laser (Power technology, Alexander AR, USA) with a wavelength of
639\, nm. The length of the laser pulses \( t_{i1} \) and the time lag between the pulses \( \Delta t \)
were set by an acousto-optical tunable filter (AOTF). The illumination intensity was
set to 3±0.3 kW/cm\(^2\) for both lasers. A circular diaphragm was introduced in the back
focal plane of the tube lens to confine the illumination area and create a flat laser illu-
mination profile. An appropriate filter combination (dichroic Z405/514/647/1064rpc
and emission filter Z515/647m, Chroma Technology, Brattleboro,USA) permitted the
detection of individual fluorophores by a liquid nitrogen cooled slow-scan CCD cam-
era system (Princeton Instruments, Trenton, NY, USA). The time between two sets
of laser pulses was limited by the readout time of the CCD chip, which was about 290 ms. A dichroic wedge diverted the emitted light to two different regions of the CCD according to emission wavelength, see Fig. 1. In this way the fluorescence signals from the Cy3B and ATTO647N molecules were separated. For the observation of the diffusion of single DNA molecules in solution, the molecules were dissolved in phosphate buffered saline (PBS: 150 mM NaCl, 10 mM Na₂HPO₄/NaH₂PO₄, pH 7.4), 5% dextran T500 / PBS or 10% dextran T500 / PBS. 1 ml of the solution was placed on a cover slip in a custom made sample holder and the focus of the microscope was set several μm into the solution (depth of focus ≈ 1 μm). The number of molecules in the focal volume was chosen so low that individual fluorophores could be resolved. For each time lag approximately 5000 images were taken (in both color channels), which resulted in the measurement of about 10000-20000 diffusion steps. For typical raw data see the supplementary movie (t₀₀ = 5 ms, Δt = 200 μs).

Figure 1: Microscope setup. Two lasers (514 nm and 639 nm) were focused on the backfocal plane of a high NA objective which resulted in widefield illumination of the sample. The fluorescence light from the red and green fluorophores respectively was split by a dichroic wedge and directed to different regions on the CCD chip. The two lasers were pulsed independently by an AOTF.
3 Derivation of theoretical result for the averaged MSD

We assume the stochastic process underlying the random walk of the observed molecules is ergodic, which means that the ensemble average \( \langle \ldots \rangle \) is identical to the time average, which is the experimentally accessible quantity. In the following the overline denotes the average over the illumination time.

\[
\text{MSD}(\Delta t) = \left\langle \left( \mathbf{r}(t + \Delta t) - \mathbf{r}(t) \right)^2 \right\rangle
\]

\[
= \left\langle \left( \frac{1}{t_{\mathrm{ill}}} \int_{-t_{\mathrm{ill}}}^{0} dt' \mathbf{r}(t + \Delta t + t') - \frac{1}{t_{\mathrm{ill}}} \int_{-t_{\mathrm{ill}}}^{0} dt' \mathbf{r}(t + t') \right)^2 \right\rangle
\]

\[
= \frac{1}{t_{\mathrm{ill}}^2} \int_{-t_{\mathrm{ill}}}^{0} dt' \int_{-t_{\mathrm{ill}}}^{0} dt'' \left\langle \left( \mathbf{r}(t + \Delta t + t') - \mathbf{r}(t + t') \right)^2 - \mathbf{r}^2(t + \Delta t + t') - \mathbf{r}^2(t + t') + \mathbf{r}(t + t') \mathbf{r}(t + \Delta t + t') \mathbf{r}(t + \Delta t + t') \right\rangle
\]

In the last step we expanded the brackets, regrouped the terms and switched the time integral with taking the ensemble average, which is admissible because taking the ensemble average is a linear operation. We further assume that the stochastic process is second-order stationary, i.e.

\[
\left\langle \mathbf{r}(\tau) \mathbf{r}(\tau') \right\rangle = \left\langle \mathbf{r}(\tau + a) \mathbf{r}(\tau' + a) \right\rangle
\]

such that

\[
\text{MSD}(\Delta t)
\]

\[
= \frac{1}{t_{\mathrm{ill}}^2} \int_{-t_{\mathrm{ill}}}^{0} dt' \int_{-t_{\mathrm{ill}}}^{0} dt'' \left\langle \left( \mathbf{r}(\Delta t + t') - \mathbf{r}(t') \right)^2 \right\rangle - \left\langle \left( \mathbf{r}(t') - \mathbf{r}(t'') \right)^2 \right\rangle
\]

\[
= \frac{1}{t_{\mathrm{ill}}^2} \int_{-t_{\mathrm{ill}}}^{0} dt' \int_{-t_{\mathrm{ill}}}^{0} dt'' \left\langle \left( \mathbf{r}(\Delta t + t'' - t') - \mathbf{r}(0) \right)^2 \right\rangle - \left\langle \left( \mathbf{r}(t'') - \mathbf{r}(t') \right)^2 \right\rangle
\]

\[
= \frac{1}{t_{\mathrm{ill}}^2} \int_{-t_{\mathrm{ill}}}^{0} dt' \int_{-t_{\mathrm{ill}}}^{0} dt'' \left( \text{MSD}(\Delta t + \varepsilon) - \text{MSD}(\varepsilon) \right) \quad \text{with} \quad \varepsilon := t'' - t' , \quad d\varepsilon = dt''
\]

For normal, two-dimensional diffusion MSD(\(\tau\)) = 4D |\(\tau\)|.

\[
\text{MSD}(\Delta t) = \frac{4D}{t_{\mathrm{ill}}^2} \int_{-t_{\mathrm{ill}}}^{0} dt' \int_{-t_{\mathrm{ill}} - |\Delta t + \varepsilon|}^{-t''} d\varepsilon \left| \Delta t + \varepsilon \right| - |\varepsilon|
\]

\[
I_1 := \int_{-t_{\mathrm{ill}}}^{0} dt' \int_{-t_{\mathrm{ill}} - t''}^{0} d\varepsilon |\varepsilon| = \int_{-t_{\mathrm{ill}}}^{0} dt' \frac{1}{2} \left| t_{\mathrm{ill}} + t' \right|^2 + \left( t'' \right)^2 = \frac{1}{3} t_{\mathrm{ill}}^3
\]
\[ I_2 := \int_{-\tau_{ii}}^{0} dt' \int_{-\tau_{ii} - t'}^{0} d\varepsilon \ |\Delta t + \varepsilon| \]

For \( \Delta t > t_{ii} \) it holds that \( \Delta t + \varepsilon > 0 \) since \(-t_{ii} < \varepsilon < t_{ii} \), so
\[ I_2 = \Delta t \ t_{ii}^2 + \int_{-\tau_{ii}}^{0} dt' \int_{-\tau_{ii} - t'}^{0} d\varepsilon \ \varepsilon = \Delta t \ t_{ii}^2 \]

For \( \Delta t \leq t_{ii} \)
\[ I_2 = \int_{-\tau_{ii}}^{0} dt' \int_{-\tau_{ii} - t'}^{0} d\varepsilon \ |z| \quad \text{with} \quad z := \Delta t + \varepsilon \quad , \quad d\varepsilon = d\varepsilon \]
\[ = \int_{-\tau_{ii}}^{\Delta t - t_{ii}} dt' \int_{-\tau_{ii} - t'}^{\Delta t} d\varepsilon \ z + \int_{\Delta t - t_{ii}}^{0} dt' \left( \int_{-\tau_{ii} - t'}^{0} d\varepsilon \ (-z) + \int_{0}^{-t' + \Delta t} d\varepsilon \ z \right) \]
\[ = -\frac{1}{3} \Delta t^3 + \Delta t^2 \ t_{ii} + \frac{1}{3} t_{ii}^3 \]

Combining these terms we arrive at
\[ \text{MSD}(\Delta t) = \frac{4D}{t_{ii}^2} (I_2 - I_1) = 4D \times \begin{cases} \frac{(\Delta t^2 - \frac{1}{3} \Delta t^3)}{t_{ii}^3} & \text{for} \quad \Delta t \leq t_{ii} \\ \frac{\Delta t}{t_{ii}} & \text{for} \quad \Delta t > t_{ii} \end{cases} \]

Due to the finite positional accuracy there is an additional constant term which equals \( 2(\sigma_{XYB}^2 + \sigma_{ATT0647N}^2) = 4\sigma^2 \), where \( \sigma_{XYB} \) and \( \sigma_{ATT0647N} \) are the one-dimensional positional accuracies for Cy3B and ATT0647N respectively. The theoretical expression derived above plus the constant offset \( 4\sigma^2 \) was fit to the experimentally obtained MSD with free fit parameters \( D \) and \( \sigma \). The fit was a weighted least-squares fit with the inverse square root of the errors of every data point as weights. On average \( \sigma = 46nm \). For the purpose of better comparability the offsets \( 4\sigma^2 \) were subtracted in Fig. 2 in the main text. The value for \( \sigma \) reported here is comprised of 1. a contribution due to the finite accuracy for the position determination by Gaussian fitting and 2. remaining chromatic aberration which has not been corrected for completely. Chromatic aberration was corrected for with an accuracy of approximately 10nm (see below).

4 Fitting of single-molecule signals

Details about finding and fitting single-molecule signals were described elsewhere [3, 4]. Briefly, raw images were filtered with a two-dimensional Gaussian whose width corresponds to the width of the point spread function (PSF) of the microscope. This procedure optimized the signal to noise ratio. The positions of the pixels whose value after filtering exceeded a certain multiple of the noise were used as initial values for the fitting of a two-dimensional Gaussian in the unfiltered image. This thresholding procedure separated true single-molecule signals from noise. From the Gaussian fit, position, width and integrated intensity of the single molecule signal were determined.
5 Correction for chromatic aberration

To achieve an exact spatial correlation between the two detection channels we imaged a fluorescent bead which could be detected in both channels. The bead was adsorbed to a coverslip and moved through the whole region of interest. The positions of the bead in the Cy3B channel \( r_i^{\text{green}} \) and the the ATTO647N channel \( r_i^{\text{red}} \) were determined as described in the previous section. To interpolate the shift for areas which had not been covered by the bead fifth-order polynomials \( f_x(r) \) and \( f_y(r) \) were fit to the measured positions (separately for the two directions \( x \) and \( y \)) such that

\[
\sum_i (f_x(r_i^{\text{green}}) - (x_i^{\text{red}} - x_i^{\text{green}}))^2 = \text{minimal!}
\]

\[
\sum_i (f_y(r_i^{\text{green}}) - (y_i^{\text{red}} - y_i^{\text{green}}))^2 = \text{minimal!}
\]

\( f_x(r) \) and \( f_y(r) \) then gave the corrective shifts – in \( x \) and \( y \) respectively – to be applied to a position \( r \) measured in the green channel. Fig. 1d in the main text shows an example for these shifts. To quantify how accurately the described procedure eliminated chromatic aberration we applied the corrective shifts \( f_x(r) \) and \( f_y(r) \) to the positions of the multifluorescent bead used to measure those shifts. From the remaining differences between the positions in the two color channels we calculated that chromatic aberration was eliminated down to a length scale of approximately 10nm.

6 Quantification of microscope stage drift

To exclude that drift of the microscope stage influenced the measurements we measured the MSD of fluorescent beads adhered to a cover slip, see Fig. 2. We found that the stage drift (diffusion coefficient \( D = 3.1 \pm 1.4 \cdot 10^{-6} \mu m^2/s \)) is negligible on the time scale relevant for our experiments (< 100ms).

7 Particle Image Cross-Correlation Spectroscopy (PICCS)

PICCS calculates the correlation function between two different types of signals termed "green" and "red" without loss of generality. The PICCS algorithm illustrated in Fig. 3 results in the cumulative correlation function \( C_{\text{cum}}(l, \Delta t) \) where \( \Delta t \) is the time-lag between the illumination of the two probes.

If, per image, there is exactly one pair of correlated signals the correlation function \( C_{\text{cum}}(l, \Delta t) \) equals \( P_{\text{cum}}(l, \Delta t) \), the probability for finding a distance smaller than \( l \) between a green and a red signal. If only for a fraction \( \alpha \) of all green signals there is a correlated red signal, we observe \( C_{\text{cum}}(l, \Delta t) = \alpha P_{\text{cum}}(l, \Delta t) \). Typically there is more than one green signal per image and therefore also more than one red
signal. If $t$ gets bigger, neighboring red signals in close proximity are counted by the PICCS algorithm although they are not correlated with the green signal. Additionally there might be red signals which are not correlated with any green signal at all. These red signals, in close proximity or not correlated with any green signal, lead to an additional contribution $c_{\text{red}} \cdot \pi t^2$ to $C_{\text{cum}}(l, \Delta t)$ — under the assumption that the positions of the red signals follow a uniform random distribution with density $c_{\text{red}}$. In total $C_{\text{cum}}(l, \Delta t) = \alpha P_{\text{cum}}(l, \Delta t) + c_{\text{red}} \cdot \pi t^2$.

If there are no red signals in addition to the ones correlated with a green one, $c_{\text{red}}$ can be calculated from the density of green signals $c_{\text{green}}$, the correlation fraction $\alpha$ and the image area $A$ by

$$c_{\text{red}} = \alpha (c_{\text{green}} A - 1) / A = \alpha (c_{\text{green}} - 1/A) \equiv c_{\text{red}}^{*}$$  

If $1/A \ll c_{\text{green}}, c_{\text{red}} \approx c_{\text{green}}$. In general $c_{\text{red}} = c_{\text{red}}^{*} + c_{\text{red,un correlated}}$, where $c_{\text{red,un correlated}}$ is the density of red signals which are not correlated with any green signal.

In practice $P_{\text{cum}}(l, \Delta t)$ is retrieved by subtraction of the linear part of $C_{\text{cum}}(l, \Delta t)$ when plotted against $l^2$ and subsequent normalization to 1, see Fig. 4.

Since the probability to find exactly the distance $l$ between a green and a red signal is $\partial P_{\text{cum}}(l, \Delta t)/\partial l$ the MSD could in principle be calculated by $\overline{\text{MSD}}(\Delta t) = \int_0^\infty dl \ l^2 \partial P_{\text{cum}}(l, \Delta t)/\partial l$. Due to noise this direct calculation would lead to large errors. Instead the heuristic formula

$$P_{\text{cum}}(l, \Delta t) = \beta \left( 1 - \exp \left( -\frac{l^2}{2 \text{sd}_1(\Delta t)^2} \right) \right) + (1 - \beta) \left( 1 - \exp \left( -\frac{l^2}{2 \text{sd}_2(\Delta t)^2} \right) \right)$$

was fit to $P_{\text{cum}}(l, \Delta t)$ with 3 fit parameters $\beta$, $\text{sd}_1(\Delta t)$ and $\text{sd}_2(\Delta t)$, which gave a good description of the data in all cases and eliminated high-frequency noise, see Fig. 4b. The MSD is then simply $\overline{\text{MSD}}(\Delta t) = \beta \text{sd}_1(\Delta t)^2 + (1 - \beta) \text{sd}_2(\Delta t)^2$. 

Figure 2: MSD of fluorescent beads adhered to a cover slip (blue circles). The data is fit by a linear diffusion model (solid black line) with a diffusion coefficient of $D = 3.1 \pm 1.4 \cdot 10^{-6} \mu m^2/s$ and a positional accuracy of 18nm.
Figure 3: PICCS algorithm. For all green signals (solid circles) the number of red signals (open circles) are counted which fall into a circle of radius $l$ from a green signal. The total number is divided by the number of green signals. By increasing $l$ from 0 to $l_{\text{max}}$ the whole correlation function $C_{\text{cum}}(l, \Delta t)$ is constructed. To avoid edge effects, only the green signals in the area bounded by the dashed line are used. Those signals lie farther away from the edges of the image than the maximal distance $l_{\text{max}}$ analyzed. Here $l_{\text{max}} = 2 \mu m$. The signal positions were simulated with these parameters: density of green signals $c_{\text{green}} = 1 \mu m^{-2}$, correlation fraction $\alpha = 0.5$ (results in a density of red signals of $c_{\text{red}} = 0.5 \mu m^{-2}$), correlation length $\sigma = 150 \, nm$.

Finally, we have to take into consideration that the positions of single-molecules can be determined only with a finite positional accuracy. The probability $P(\xi, \eta, \Delta t)$ to observe two correlated signals separated by a vector $(\xi, \eta)$ is the convolution of the real probability $P_{\text{real}}(\xi, \eta, \Delta t)$ and the probability density $P_{\text{pos.acc.}}(\xi, \eta)$ describing the (apparent) correlation due to the finite positional accuracy [5].

$$
P(\xi, \eta, \Delta t) = \int \int d\xi' d\eta' P_{\text{real}}(\xi - \xi', \eta - \eta', \Delta t) P_{\text{pos.acc.}}(\xi', \eta')$$

$$P_{\text{pos.acc.}}(\xi, \eta) = \frac{1}{2\pi\sigma^2} \exp \left( -\frac{\xi^2 + \eta^2}{2\sigma^2} \right)$$

where $\sigma = \sqrt{2}\sigma_{\text{pos.acc.}}$ and $\sigma_{\text{pos.acc.}}$ is the one-dimensional positional accuracy. For
simplicity we assume here that the positional accuracy is the same for both types of signals. The cumulative probability $P_{\text{cum}}(l, \Delta t)$ is then found by integration of $P(\xi, \eta, \Delta t)$ in polar coordinates

$$P_{\text{cum}}(l, \Delta t) = \int_0^l dr \int_0^{2\pi} d\phi P(r, \phi, \Delta t)$$

(4)

with $r = \sqrt{\xi^2 + \eta^2}$, $\phi = \arctan(\eta/\xi)$.

The MSD calculated from this cumulative probability has a constant contribution $4\sigma^2$ [5] as already mentioned above.

8 Error determination

The errorbars in Fig. 2 in the main text were determined from Monte Carlo simulations of the probability $P_{\text{cum}}(l, \Delta t)$ with exactly the same parameters as found in the experiments and the number of molecule positions actually recorded. For each simulation the MSD was determined as described in the previous section and the standard deviation calculated from 100 simulations run with identical parameters.

9 FCS measurements

FCS measurements were performed on a home-made confocal microscope described in detail elsewhere [1]. The measured auto-correlation curves $G(\tau)$ were fit to a model for 3D diffusion.

$$G(t) = \frac{G_0}{(1 + \tau/\tau_d) \sqrt{1 + (a^{-2})(\tau/\tau_d)}}$$

with the free parameters $G_0$, the auto-correlation amplitude, $a$, a geometric factor equal to the ratio of width and height of the focal volume, and $\tau_d$, the diffusion time. First, this expression was fit to the auto-correlation curves for the three different types of solvents (PBS, 5% dextran, 10% dextran) leaving all parameters free. Then parameter $a$ was fixed to the average over the values obtained from the 3 auto-correlation curves. Finally, Eq. 9 was fit again to all auto-correlation curves with $G_0$ and $\tau_d$ as free parameters, which led to the ratios of diffusion times reported in the main text. Errors were determined as errors of the fit of the model to the measured curves. Fig. 5 shows the measured auto-correlation curves and fits of Eq. 9.

10 Viscosity measurements

Bulk kinematic viscosity measurements were performed with a Cannon-Ubbelohde viscometer (CANNON Instrument Company, State College, PA, USA). Measurements of all different solvents used were conducted in triplicates and the error determined as the standard deviation.
References


Figure 4: Examples for (a) $C_{\text{cum}}(l, \Delta t)$ and (b) $P_{\text{cum}}(l, \Delta t)$ found in experiments with $\Delta t = 0.3\text{ms}$, $t_{\text{ill}} = 3\text{ms}$. The solid black line in (a) is a linear fit to the linear part of $C_{\text{cum}}(l, \Delta t)$ plotted against $l^2$. This linear contribution is subtracted and the resulting curve is normalized to one to obtain $P_{\text{cum}}(l, \Delta t)$ shown in (b). The solid black line in (b) is a fit of Eq. 2 to the data.
Figure 5: Auto-correlation curves measured by FCS for the DNA oligomer in PBS (black), 5% dextran (blue) and 10% dextran (red). The solid lines are fits of Eq. 9 to the measured auto-correlation curves with $G_0$ and $\tau_d$ as free parameters. For better comparability all auto-correlation curves were normalized to the fitted $G_0$. 