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A polarized view on DNA under tension

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In the past decades, sensitive fluorescence microscopy techniques have contributed significantly to our understanding of the dynamics of DNA. The specific labeling of DNA using intercalating dyes has allowed for quantitative measurement of the thermal fluctuations the polymers undergo. On the other hand, recent advances in single-molecule manipulation techniques have unraveled the mechanical and elastic properties of this intricate polymer. Here, we have combined these two approaches to study the conformational dynamics of DNA under a wide range of tensions. Using polarized fluorescence microscopy in conjunction with optical-tweezers-based manipulation of YOYO-intercalated DNA, we controllably align the YOYO dyes using DNA tension, enabling us to disentangle the rapid dynamics of the dyes from that of the DNA itself. With unprecedented control of the DNA alignment, we resolve an inconsistency in reports about the tilted orientation of intercalated dyes. We find that intercalated dyes are on average oriented perpendicular to the long axis of the DNA, yet undergo fast dynamics on the time scale of absorption and fluorescence emission. In the overstretching transition of double-stranded DNA, we do not observe changes in orientation or orientational dynamics of the dyes. Only beyond the overstretching transition, a considerable depolarization is observed, presumably caused by an average tilting of the DNA base pairs. Our combined approach thus contributes to the elucidation of unique features of the molecular dynamics of DNA.

INTRODUCTION

With the advent of single-molecule observation techniques, DNA has become a model system for studying the dynamics of semi-flexible polymers.1–4 The direct visualization of fluorescently labeled DNA undergoing Brownian motion using DNA-specific fluorescent dyes has allowed for a quantitative understanding of its thermal fluctuations.5–7 Many dyes specific to double-stranded DNA (dsDNA) have been employed in fluorescence applications both in bulk and at the microscopic level. These dyes can be classified according to their mode of binding to DNA into two major categories: groove binding dyes (e.g., DAPI or Hoechst, binding in the minor groove) and intercalating dyes (e.g., ethidium bromide, SYBR dyes, or cyanine dyes such as TOTO, YO, and YOYO). The latter are planar organic structures, allowing in the minor groove) and intercalating dyes (e.g., ethidium bromide, SYBR dyes, or cyanine dyes such as TOTO, YO, and YOYO). The latter are planar organic structures that specifically bind dsDNA by sandwiching in between consecutive base pairs [Fig. 1(a)], thereby lengthening the DNA upon binding.8,9 Both groove binders and intercalators have a well-defined orientation with respect to the helix axis of the DNA. For intercalators, the absorption and emission dipole moments are roughly perpendicular to the DNA helix axis.10,11

This latter property makes intercalated dyes particularly suitable for polarized optical spectroscopy.12 Such techniques, including linear or circular dichroism spectroscopy and fluorescence anisotropy, can be used to study the orientation as well as the orientational dynamics in macro- or microscopic samples.12–14

Fluorescence polarization anisotropy (FPA) measures the reorientation of a sample within the ultrashort time span between excitation and emission of a fluorescence photon. This is achieved by exciting the sample with linearly polarized light and probing how much fluorescence signal is emitted with its polarization parallel and perpendicular to the excitation polarization. The anisotropy r is defined as

\[ r = \frac{I\parallel - I\perp}{I\parallel + 2I\perp}, \]

where the I terms correspond to the fluorescence (emission) intensities with polarization parallel and perpendicular to the excitation polarization, indicated by the suffixes. Polarization anisotropy has been used to study reorientations of DNA conformations both in steady state15,16 and in a time-resolved manner17 for quantitation of rotational diffusion.

Linear-dichroism (LD) measurements, on the other hand, probe the anisotropic absorption of linearly polarized light. LD measurements require sample orientation: for unordered, isotropic samples, the absorbing molecules on average assume every orientation with equal probability, rendering LD zero. LD is defined as

\[ LD = \frac{\parallel A - \perp A}{\parallel A + \perp A}, \]

where the prefixes indicate the incident light polarization with respect to the sample orientation axis. To avoid the comparison of absolute sample

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absorptions, one uses the reduced linear dichroism $LD_r$, i.e., the $LD$ normalized by the isotropic absorption $A_{iso}$. For a sample with cylindrical symmetry around the sample axis, this can be estimated as $A_{iso} = \frac{1}{2}(AA + 2AA)$. For fluorescent samples, $LD_r$ can also be measured by probing the fluorescence emission upon polarization excitation,

$$LD_r = \frac{I - I_{iso}}{I_{iso}} = \frac{I - I_{iso}}{\frac{1}{2}(I + I_{iso})}, \quad (2)$$

Several techniques exist to impose a macroscopic preferred sample orientation required for $LD_r$, including the use of flow fields, electrophoresis, compressed gels, stretched films, or wet spinning. Values for $LD_r$ yield a measure for the average orientation of the absorbing (and, for fluorescence-detected $LD_r$, also emitting) dipole,

$$LD_r = \frac{3}{2}S \left(3\cos^2\theta - 1\right), \quad (3)$$

with $\theta$ being the average angle between the absorbing dipole moment and the sample orientation, and $S$ being the orientation factor, equal to 1 for a perfectly aligned sample and 0 for an isotropic one. In practice, sample alignment is imperfect, such that $LD_r$ measurements yield a measure for $\left<\cos^2\theta\right>$, i.e., an average effective dipole orientation angle. This makes the quantitative interpretation of $LD_r$ measurements problematic: imperfect sample alignment always yields an underestimated $\theta$ if no independent assessment of $S$ can be made.

$LD_r$ measurements have been widely applied to DNA and DNA-binding (intercalating) dyes in bulk spectrometric assays. Due to the aforementioned difficulties, some reports do not agree on the orientation of the base-pairing plane of dsDNA, assessed using $LD_r$ spectroscopy in the UV, where nucleic acids strongly absorb. Inclination angles of up to 25° have been reported[11,14,19,20] whereas structural studies reported the base-pairing plane to make only a small (<10°) angle with respect to the B-form helix. A similar disagreement exists about the orientation of fluorescent dyes intercalated in dsDNA.[10,11,14,20–24] In addition to spectrometric assays, polarized fluorescence microscopy has been applied to the study of intercalated dye orientation. In these cases, samples were oriented using hydrodynamic flow. However, the discrepancy about intercalator orientation remained unresolved. In one study, $LD_r$ was measured on intercalated DNA extended using optical tweezers by exciting with two perpendicular polarizations and detecting total fluorescence. From these data, the conclusion was drawn that the absorption dipole moments of the intercalator YOYO makes an angle of \(\sim 78°\) with the DNA long axis. This study, however, neglected the rotational dynamics of the dipoles and energy transfer between YOYO’s and chromophores (see below).

Fluorescence anisotropy and linear-dichroism measurements can also be united by combining polarization-selective excitation with polarization-sensitive detection of the fluorescence of oriented samples. This yields four intensity measurements, $ex I_{em}$, where the prefix "ex" indicates the excitation polarization and the suffix "em" indicates the fluorescence polarization. To allow a quantitative comparison of the (most often uncalibrated) intensities $ex I_{em}$, the following emission ($P$) and absorption ($Q$) polarization ratios are often considered instead:

$$P_\parallel = \frac{\| I_{\parallel}^{\parallel} \| - \| I_{\perp}^{\parallel} \|}{\| I_{\parallel}^{\parallel} \| + \| I_{\perp}^{\parallel} \|}, \quad P_\perp = \frac{\| I_{\parallel}^{\perp} \| - \| I_{\perp}^{\perp} \|}{\| I_{\parallel}^{\parallel} \| + \| I_{\perp}^{\perp} \|},$$

$$Q_\parallel = \frac{\| I_{\parallel}^{\parallel} \| - \| I_{\perp}^{\parallel} \|}{\| I_{\parallel}^{\parallel} \| + \| I_{\perp}^{\parallel} \|}, \quad Q_\perp = \frac{\| I_{\parallel}^{\perp} \| - \| I_{\perp}^{\perp} \|}{\| I_{\parallel}^{\parallel} \| + \| I_{\perp}^{\parallel} \|}.$$  

These $P$ and $Q$ ratios are related to $r$ and $LD_r$, respectively [cf. Eqs. (1) and (2)]

The application of single-molecule manipulation techniques to DNA provides fine control over its extension, while allowing measurements of the pico-Newton forces involved in stretching it. Using such techniques, the mechanical and elastic properties of DNA have been assessed and are now well understood. Here, we have taken an approach combining optical tweezers and polarized fluorescence microscopy, to investigate the dynamics of intercalated DNA under increasing levels of tension. We directly measured the alignment of the transition dipoles of the dyes when the DNA is pulled taut. We performed Monte Carlo simulations of partially extended polymers. Moreover, we extended the analysis of Irving to express the polarization ratios $P$ and $Q$ in terms of the orientation of individual dyes with respect to a laboratory symmetry axis, originally developed for the study of the orientation of myosin light chains in muscle fibers. In doing so, we are able to discern between fluorescence depolarization due to
either statically tilted intercalation or fast diffusional dynamics of DNA and dyes. Moreover, by pulling with forces at which the DNA undergoes the overstretching (OS) transition, we study the dynamics of DNA undergoing force-induced base pair melting. We show that intercalating dyes stay bound to even largely melted DNA.

MATERIALS AND METHODS

Optical trapping and polarized fluorescence microscopy

The optical-trapping instrument was adapted from that described before (Fig. 4 of the supplementary material). For polarized fluorescence excitation, a linearly polarized 473-nm laser (Cobolt Blues™, 25 mW cw, Cobolt AB, Stockholm, Sweden) was led through an electro-optic modulator (EOM, model 350-80, Conoptics, Danbury, CT). The polarization state of the excitation laser was modulated by applying different sequential DC voltages to the EOM synchronized with camera readout (see below). A Berek variable wave plate (model 5540, New Focus) was used for fine-tuning the polarization state after the EOM. The polarized light was decollimated for widefield excitation and coupled into the microscope objective using a dichroic mirror (“DM3”: z473rdc, Chroma).

Fluorescence emission was bandpass filtered (“EM1”: hq540/80m-2p, Chroma) and imaged onto a sensitive electron-multiplying CCD camera (“EM-CCD”: Cascade 512B, Princeton Instruments, Monmouth Junction, NJ). In front of the camera, a Wollaston prism (WSP9015, FOCTek Photonics, Fujian, China) was placed to spatially decompose the fluorescent light upon polarization onto different segments of the CCD sensor.

The custom-built flow cell featuring multiple parallel, laminar channels with different buffers that can be swiftly exchanged has been described before.

Procedures for polarized fluorescence measurements

To measure the four polarized intensities $I_{ex}$, the excitation polarization was alternated using the EOM, while the emission signal was split into two perpendicular emission polarizations using the Wollaston prism, imaged simultaneously onto the camera [Fig. 4(a) of the supplementary material]. Camera readout, excitation shutter polarization, and trap movement were synchronized using computer-generated signals (PCI-6229 multifunction DAQ-board, National Instruments) as follows [Fig. 4(b) of the supplementary material]. The 473-nm laser shutter was opened with a transistor-transistor logic (TTL) signal. Concurrently, the camera was triggered for 1-s exposure of one frame by the rising flank of a TTL pulse. Next, the excitation polarization was rotated by 90° by changing the bias voltage ($V_{\perp} \rightarrow V_{\parallel}$) on the EOM and exposure of a second frame was started by another TTL pulse to the camera. After the second frame, the shutter was closed and the polarization state was changed back. In the interval to the next exposure cycle, the DNA molecule was stretched or released by stepwise displacement of one of the traps using a linear actuator (T–LA28, Zaber Technologies, Inc., Richmond, BC, Canada). The emission splitting using the Wollaston prism allowed for simultaneous imaging of the two polarizations onto separate areas of the CCD sensor.

The camera synchronization signal was recorded in conjunction with the force on the DNA and the bead-bead distance. Hence, all fluorescence images were associated with the corresponding DNA tension and extension. Fluorescence intensities were obtained from the images as follows. In every frame, the total fluorescence intensity in both polarizations was extracted by summing the intensities in a rectangle, with a fixed height of typically 8 pixels (corresponding to ~1.4 µm) perpendicular to the DNA long axis and a width scaling with the corresponding DNA extension, to encompass the entire DNA molecule. For background correction, the average intensity in a dark region shifted several pixels with respect to the DNA was subtracted from each pixel. As a consistency check, we repeated the above procedure for segments of the imaged DNA molecules (by changing the width of the rectangular box), which yielded the same (normalized) intensities.

Sample preparation of DNA and dyes

Biotinylation of bacteriophage lambda DNA was performed as described elsewhere. Unlabeled DNA molecules were captured between a set of trapped beads and stretched to measure the contour length. Next, the bead-DNA-bead complex was moved to a 10-mM Tris buffer containing 100–200 nM YOYO (trade name YOYO-1, Molecular Probes) and 5–10 mM NaCl for intercalation. We have found this relatively low salt concentration to enhance the binding stability of YOYO. The final labeling ratio was set both by the time duration the molecule was exposed to YOYO and by the DNA tension at which the labeling took place. For each molecule, this ratio was determined by comparing its contour length after intercalation with that before it, assuming every dye moiety to add 0.34 nm to the DNA contour length.

Data fitting and analysis

The dependence of the $P$ and $Q$ polarization ratios on relative DNA extension was fitted to the different models described in Secs. 2 and 3 of the supplementary material. For a range of angles that describe the orientation and mobility of the dyes (see Fig. 2 and below), values of $P/Q$ were tabulated as a function of $L/L_0$. The fit function, used for Levenberg-Marquardt least-squares fitting, calculated function values by linearly interpolating these tabulated values. An angle $\beta$ (Sec. 3 of the supplementary material) was used to account for energy...
FIG. 2. Coordinate system for polarized fluorescence of DNA under controlled tension. The DNA is pulled taut using two optical traps on the y-axis. The fluorescence excitation propagates in the positive z-direction with its polarization alternately aligned with the x- and y-axis (⊥ and ||, respectively); emission is collected in epi-fluorescence mode, i.e., in the negative z-direction. The absorption and emission dipole moments of the intercalated dyes (depicted as cylindrical disks) make an angle θ with respect to the local DNA helix axis. In the “fast wobble” model (see text), the dye undergoes rapid reorientations at the time scale between absorption and emission of a photon within a cone of semi-angle δ. The two intercalated chromophores of a YOYO dimer make an azimuthal angle β with respect to each other, as indicated by the cylinder on the right. The latter becomes relevant when energy transfer between these chromophores is accounted for.

transfer between the two YOYO chromophores. This angle was determined to be 51°, based on one bis-intercalating dye dimer sandwiched two base pairs and every chromophore unwinding the DNA helix by ~20°. In the case of energy transfer between the chromophores, the emitted photon is equally likely emitted by either of the two. Therefore, such energy transfer can be emulated in the simulations by setting β = 51°/2 = 25° as the effective azimuthal angle between the absorbing and emitting dipoles. In practice, the two chromophores of a YOYO dimer are indeed close enough such as to allow efficient transfer.

In the least-squares fits, β was kept fixed at either 0° or 25°, whereas the inclination angle θ and the cone angle δ (Sec. 3 of the supplementary material) were fit parameters. Fits were performed globally, i.e., on all four polarization ratios P||, P⊥, Q||, and Q⊥ simultaneously.

RESULTS AND DISCUSSION

Force-extension before and after labeling

Unlabeled DNA molecules were suspended between two optically trapped beads. Force-extension analysis was used to reveal whether a single DNA molecule was captured, upon which the DNA was moved to a buffer containing 100–200 nM YOYO. For DNA molecules under tension, YOYO binding could be observed as a decrease of tension, caused by the unwinding of the DNA upon intercalation. The extent of labeling could be controlled simply by changing the duration of exposure to dye. Figure 1(b) shows force-extension graphs for DNA molecules before and after labeling. The extent of labeling could be calculated for each DNA molecule from the ratio of contour length before and after labeling. Typically, the ratio of dyes to base pairs was 1:40. At higher labeling ratios (up to 1:10), the DNA overstretching transition, occurring as a force plateau at 65 pN, picks up a slope (cf. gray traces in Fig. 1(b)). This is most likely caused by the intercalating dyes interfering with the transition, rendering it less cooperative, and causing the end of the transition to become less sharp, as will be discussed below. In all cases, the labeling ratio was ensured to be low enough to preclude YOYO binding modes other than intercalation. Note that under these conditions, YOYO does not bind to single-stranded DNA (including melting bubbles) or S-DNA, but only to double helical dsDNA.

Polarized fluorescence

Next, the DNA was moved to a buffer channel without YOYO. Here, the concurrent polarized fluorescence and DNA extension experiments were performed by recording fluorescence images with polarization of the excitation light alternating between perpendicular and parallel to the DNA long axis, respectively. These orthogonal axes are depicted in Fig. 2. A Wollaston prism in front of the camera (see Fig. 4 of the supplementary material) was used to spatially separate the emitted fluorescence into two images with orthogonal polarizations to generate four intensity signals for every DNA extension, ε0/em, with excitation polarization “ex” and emission polarization “em” being either parallel (||) or perpendicular (⊥) to the DNA long axis. After recording images for both polarizations “ex,” the DNA extension was stepwise increased. Figure 3(a) shows a sequence of images of the four intensity channels ε0/em for different DNA extensions and forces. One can easily observe that the dyes gradually align their emission dipoles in the direction orthogonal to the DNA, as qualitatively evidenced by the fading of channels of those with a parallel component, in favor of I⊥. This effect is more readily seen when the fluorescence in each frame is integrated and background corrected (see the section titled Materials and methods). Figure 3(b) displays the intensities I|| and I⊥ (normalized to the maximum of I||) as a function of the relative extension L/L0. The graph shows the data of 22 DNA molecules (gray symbols) as well as the same data after applying a median filter (solid gray lines) and a box average (white symbols; error bars represent the standard error of the mean). The other two channels, I∥ and I⊥, follow the same trend as I|| (not shown). For a quantitative analysis, the obtained intensities were corrected for the unequal transmission of the microscope optics for the two polarizations. In addition, the emission depolarization resulting from the collection of off-axis rays by the high numerical aperture (NA) objective was compensated for (Sec. 3 of the supplementary material).

For a quantitative interpretation of the intensities ε0/em, we used the emission and absorption polarization ratios P and Q in Eqs. (4) and (5). Figure 4 shows these polarization ratios, calculated from the four polarized intensity channels ε0/em after applying the above corrections, versus the relative extension L/L0, as well as median and box-averaged data. (The enhanced scatter in Q|| at larger extensions is caused by values of I|| and I⊥ being close to zero.)

Three regimes can be distinguished [separated by the vertical dashed lines in Figs. 4(a) and 4(b)]: the ratios incline at extensions at which the DNA is slack (L/L0 < 1; regime [i]), display a plateau in the DNA overstretching transition (1 < L/L0 ≤ 1.55; regime [ii]), and show a sharp depolarization at extensions beyond this transition (regime [iii]). These three regimes are assessed in more detail below.
FIG. 3. Polarized fluorescence of DNA at increasing extensions. (a) Consecutive polarized fluorescence images of a DNA molecule at the three extensions and forces indicated on the left. For every extension and force, the corresponding four polarized fluorescence images $I_{\text{em}}^\parallel$ and $I_{\text{em}}^\perp$ are shown. The intensities with a parallel component are decreasing with tension, in favor of $I_{\text{em}}^\perp$. (b) Integrated intensities $I_{\text{em}}^\parallel$ and $I_{\text{em}}^\perp$ (normalized to the maximum of $I_{\text{em}}^\perp$) versus the relative extension $L/L_0$. The data of 22 separate experiments on individual DNA molecules are shown (gray symbols), as well as the same data after applying a median filter (solid gray lines) and a box average (white symbols; error bars represent s.e.m.). The other two channels, $I_{\text{em}}^\perp$ and $I_{\text{em}}^\parallel$, follow qualitatively the same trend as $I_{\text{em}}^\parallel$. Beyond the transition, a sharp decline of $I_{\text{em}}^\perp$ is observed, while the intensity channels with a parallel component slightly recover. This latter fact precludes the drop in $I_{\text{em}}^\perp$ as being (exclusively) due to dye dissociation.

Regime [i]: Slack DNA

At extensions $L/L_0 < 1$, i.e., when the DNA is still slack and therefore the tension on the DNA is small, the polarization ratios show an increasing depolarization with decreasing DNA extension. This observation immediately underscores the necessity for proper sample alignment in polarized absorption measurements such as linear dichroism. For a quantitative interpretation of $LD_r$, one often assumes a perfect sample alignment [in which $S = 1$, Eq. (3)]. The behavior of the polarization ratios up to $L/L_0 = 1$ immediately exposes the vulnerability of this quantification for imperfect sample alignment, as will be discussed below. The origin of this depolarization is an increase in (thermally excited) DNA diffusion on the millisecond time scale: the lower the end-to-end length, the more conformational states the DNA can assume. Hence, at lower extensions, the dipole moments of the intercalated dyes will undergo larger thermal fluctuations around the symmetry axis connecting the two beads.

In addition to this depolarization caused by the relatively slow DNA fluctuations (“slow wobble”), the dyes may perform rapid, intrinsic dynamics on the nanosecond time scales between fluorescence excitation and emission. Such “fast wobble” also causes depolarization of the fluorescence emission. However, most polarization-based techniques cannot discriminate properly between such slow- and fast-wobble depolarization.

Irving developed a framework to express the polarization ratios $P$ and $Q$ in terms of the orientation of individual dyes with respect to a laboratory symmetry axis. He derived expressions for two cases: (1) the absorption and emission dipole vectors of the dye making a static inclination angle $\theta_a$ and $\theta_e$ to the symmetry axis (i.e., no fast wobble) and (2) the dye undergoing rapid dynamics within a cone of semi-angle $\delta$ on the time scale between absorption and emission of a photon (cone-restricted fast wobble). The angles $\theta$ and $\delta$ are graphically depicted in Fig. 2. Both a static inclination angle of the dye’s dipole moments with respect to the symmetry axis and cone mobility have a depolarizing effect on the measured $P$.
and $Q$ ratios. Note that for intercalating chromophores, the absorption and emission dipole moments have been reported to coincide: $\theta_a = \theta_e$.

The maximum $P$ and $Q$ values, attained at $L/L_0 = 1$ (where the DNA is taut and its alignment thus maximal), can be used to estimate the average dye orientation within both the static inclination model and the cone dynamics model. For the extremum values, the static inclination model corresponds best to an angle $\theta$ of $\approx 75^\circ$ with respect to the DNA axis. Likewise, in the cone dynamics model, a cone angle $\delta$ ranging between $0^\circ$ and $35^\circ$ can be found for an inclination angle $\theta$ ranging from $75^\circ$ to $90^\circ$. However, as with other methods, there is no way to discriminate between these models based only on single polarization ratio values.

In order to quantitatively interpret the dependence of the polarization ratios on DNA extension and exploit our controlled experiments of increasing slow-wobble depolarization at $L/L_0 < 1$, we performed Monte Carlo simulations of DNA conformations with fixed ends (Sec. 1 of the supplementary material). Together with a generalization of Irving’s model to allow for a local symmetry axis other than a global laboratory axis (Secs. 2 and 3 of the supplementary material), these allowed us to calculate the expected dependencies for Irving’s orientational dynamics models discussed above.

Figure 5 shows a zoom-in on regime [i] of the polarization ratios, along with four such model traces. The thick, green lines correspond to static inclination angles (i.e., no fast wobble; $\delta = 0^\circ$) of $75^\circ$ (dashed lines) and $90^\circ$ (solid lines), both of which have been reported for YOYO and other intercalators. Both these curves show a significant deviation from both our $P_i$ and $Q_i$ data. We infer that the additional depolarization induced by DNA dynamics is inconsistent with a model based on static dipole inclination alone.

The thick, red lines in Fig. 5 correspond to least-squares fits of our data to a model including fast wobble in a cone with semi-angle $\delta$ (Sec. 3 of the supplementary material). The fits were performed globally, i.e., to all four traces simultaneously. The inclination angle $\theta$ is in both cases found to be $90^\circ$; the cone angle is $\delta \approx 25^\circ$. The difference between the dashed and solid lines is the introduction of a third relevant angle: the azimuthal angle $\beta$ between the two consecutive chromophores of a bis-intercalating YOYO dimer (Fig. 2). This angle can be used to account for the energy transfer between these chromophores, which depolarizes $P$ and $Q$ as well. In this case, an effective angle of $25^\circ$ is assumed between the absorbing and emitting dipoles. The solid line represents a fitted curve where we have fixed $\beta = 25^\circ$.

The significant improvement of the fit (both with and without accounting for energy transfer) indicates that there is no static inclination of the intercalated dipole moments (i.e., $\theta = 90^\circ$) and that even when slow wobble is suppressed by pulling the DNA taut, the dyes still undergo fast reorientations, which can be described as diffusion in a cone with a semi-angle of $\approx 25^\circ$. Again, the decline of the polarization ratios for extensions below $L/L_0 = 1$ emphasizes the importance of proper sample alignment in linear-dichroism measurements, as further discussed below.

Although these conclusions hold for the internal motion of the intercalated dyes, it seems reasonable to assume that the base pairs of the DNA undergo similar dynamics themselves, permitting the dyes to wobble in their binding pockets. We will address this in more detail later.

Regime [ii]: Partially overstretched DNA

In regime [ii], the four polarization ratios stay approximately constant at the level attained at $L/L_0 = 1$. This range of
extensions coincides with the overstretching (OS) transition, in which dsDNA is progressively melted to yield two essentially single-stranded DNA (ssDNA) molecules under low-salt conditions.\textsuperscript{35,42,45} What does our observation of constant polarization ratios during this force-induced melting imply? First, it shows that in the OS transition, no significant base pair tilting occurs at or around the intercalated base pairs, which had been suggested as one of the possible structures of (partially) overstretched DNA.\textsuperscript{36} Note that the presence of intercalating moieties influences the cooperativity of the OS transition.\textsuperscript{41,44}

The otherwise extensive processivity of melting\textsuperscript{42} will presumably be limited to domains between two YOYO dyes. Nonetheless, the dyes do not appreciably start to exhibit more (“fast” or “slow”) orientational dynamics during overstretching, which would induce a steady depolarization at increasing DNA extensions. Surprisingly, despite the significant change in local structure and mechanics that accompanies the force-induced melting of DNA, no static reorientation of intercalated dyes or any alteration of their rotational dynamics is observed.

Another interesting observation is that YOYO is not forced to dissociate during the OS transition, as evidenced from the constant intensity in $I_{⊥}/I_{∥}$ [Fig. 3(b)]. Possibly, intercalated YOYO dimers locally reinforce the DNA, preventing the transition from progressing processively. Since YOYO binds with high specificity to dsDNA compared to ssDNA,\textsuperscript{42,47} the stable binding of YOYO in the OS transition suggests that at least the local DNA structure around the dyes resembles that of dsDNA.

**Regime [iii]: DNA beyond overstretching**

Unlike in the OS transition itself, the dyes do show a significant depolarization beyond the force-induced melting (regime [iii] with $L/L_0 \gtrsim 1.55$, see Fig. 4). This implies, not unexpectedly, that a considerable conformational change occurs. Surprisingly, only moderate dye dissociation occurs even in the regime where the force on the DNA increases steeply. For example, the molecule in Fig. 6(a), first pulled to 115 pN and then relaxed, displays a decrease in $I_{⊥}/I_{∥}$ of only 20% in the OS plateau before and after the pull. Figure 6(a) shows how in the molecule, which was contiguously labeled before the pull (top image), YOYO dissociates at high force only from a confined patch close to the right bead (middle image), whereas the fluorescence intensity along the rest of the molecule is retained (cf. bottom and top images). Unfortunately, most DNA molecules broke prematurely, presumably caused by photocleavage due to bleaching YOYO dyes,\textsuperscript{48,49} before getting to this stage. However, the molecules that did survive the OS transition (3 out of 22) indeed show that no massive, homogeneous YOYO dissociation occurs. This is corroborated by the observation that the three intensity channels with a “parallel” component gain intensity in the regime beyond the OS transition [e.g., $I_{∥}$ Fig. 3(b)].

What could be the nature of the orientational change that causes the depolarization beyond the OS transition? We distinguish two possibilities, which are schematically depicted in Fig. 6(b). First, the dyes may undergo enhanced (fast or slow) dynamics within their binding pockets, rendering their orientation distribution increasingly isotropic [Fig. 6(b): “wobbling bases”]. Such enhanced dynamics causes the polarization ratios to vanish, as observed. Second, the dyes may display an overall quasi-static conformational change. For example, the bases may tilt with respect to their initially orthogonal orientation as suggested before,\textsuperscript{36} due to shear forces propagating through the largely melted DNA as it is pulled from its two opposite 3′ ends. Such “static” tilt would similarly cause the observed depolarization [Fig. 6(b): “tilting bases”]. It is difficult to decisively conclude which of these two modes underlies the observed depolarization. We would, however, expect a condition of enhanced conformational dynamics to quench fluorescence emission, similar to what is observed for intercalating dyes free in solution, which has been attributed to efficient quenching caused by the internal motion of the chromophores.\textsuperscript{50,51} Since the fluorescence pathways seem to be not considerably quenched overall (cf. the brightening of the three parallel intensity channels), we argue that a static tilting model is more plausible. Polarization measurements sensitive at the single-intercalator level will be required under these conditions to differentiate between the two possibilities.

A final interesting observation is that for intercalated DNA, the end of the overstretching transition is less sharp and that it occurs at lower extension than without intercalating dyes. For non-intercalated dsDNA, the OS transition ends at $L/L_0 = 1.7$, whereas the pulling force for intercalated DNA starts to deviate from the overstretched plateau around $L/L_0 = 1.55–1.6$, depending on the labeling ratio [Fig. 1(b)]. We argue that the melting transition is less cooperative for intercalated DNA and confined to domains between intercalators, leading to the observed more gradual switch to the enthalpic stretching phase beyond the transition, before the molecule is melted throughout.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Fig6.png}
\caption{YOYO stays bound beyond the overstretching transition. (a) Three snapshots of a molecule ($I_{∥}$ channel) before, during, and after the steep force-increase phase beyond the OS transition. The white arrow marks a short segment where YOYO disappears beyond the transition. However, the overall fluorescence intensity, which is reduced in the middle image, is regained in the bottom one, showing that YOYO is not forced to dissociate from the largely melted DNA apart from the marked segment. (b) Models explaining fluorescence depolarization beyond the OS transition. Partially overstretched DNA still contains large base-paired segments.\textsuperscript{42} Presumably, only isolated domains surrounding intercalated dyes are still base paired beyond overstretching. This may either allow the dyes to perform larger-amplitude dynamics (“wobbling bases”) or induce a net shear force on the DNA, tilting the bases and the dyes.}
\end{figure}
Comparison to $LD_r$-based studies

As mentioned before, most previous studies on the orientation of intercalating dyes, both in photospectrometric\textsuperscript{10,11,14,20,24} and in microscopy assays\textsuperscript{13,25–28} rely on measurements of the (reduced) linear dichroism ($LD_r$). Our control over the DNA alignment directly reveals the vulnerability of such approaches for imperfect alignment. Following Eq. (3), precise knowledge of the orientation factor $S$ is a prerequisite for a quantitative interpretation of $LD_r$. To underscore this importance, we show in Fig. 7 the dependence of $LD_r$ on the extension of our DNA molecules. Again, data for 22 molecules are merged. The (fluorescence-detected) $LD_r$ were calculated from the four intensity channels according to

$$LD_r = \frac{I_{em} - I_{iso}}{I_{em}} = \frac{1}{2} \left( \frac{I_{em} - I_{em}^⊥}{I_{em} + 2I_{em}^⊥} \right),$$

adding the emissions “em” in the parallel and the perpendicular channels [cf. Eq. (2)]. The (static) inclination angle $\theta$, calculated from $LD_r$, according to Eq. (3) and assuming $S = 1$, is plotted in the bottom graph. The figure immediately shows that the value of $LD_r$ or $\theta$ strongly depends on the extension of the DNA, as addressed before: Fig. 7 shows how an underestimated value is obtained at extensions $L/L_0 < 1$. We argue this to be the cause of the reported orientation angles deviating significantly from $90^\circ$\textsuperscript{10,25–28}

Moreover, steady-state linear-dichroism measurements do not allow for distinguishing between static dye inclination and depolarization due to fast wobble, like we do in Fig. 5. The thick, light gray lines in Fig. 7 correspond to Monte Carlo simulations using a static tilted inclination of $\theta = 75^\circ$ (green dotted lines) and a fast-wobble cone of $\delta = 25^\circ$ around an inclination angle $\theta = 89^\circ$ essentially perpendicular to the DNA long axis (red solid lines) as we obtained from our fits. The largely overlapping curves show that these qualitatively different models yield indistinguishable $LD_r$. Note that the values for $LD_r$ we obtained are very similar to those obtained before on YOYO-intercalated DNA extended with optical tweezers.\textsuperscript{29} That study, however, did not employ polarization-selective fluorescence detection, making it impossible to decide between these different models. Our approach of polarized excitation in combination with polarization-selective fluorescence detection on micromanipulated, individual DNA molecules indeed allows us to discriminate between these models (Fig. 5), without the need for time-resolved fluorescence measurements.\textsuperscript{17,52} The observed fast dye reorientation dynamics may also be the cause of reported tilted inclination angles of the DNA base pairs themselves within the helix, as discussed in the Introduction. Our present study may help and identify the source of this discrepancy.

CONCLUSIONS

In summary, we have demonstrated the application of single-molecule manipulation techniques to polarized fluorescence microscopy of individual DNA molecules. By extending DNA molecules in a controlled manner, we increasingly suppress Brownian dynamics, pulling the DNA taut. In combination with Monte Carlo simulations, this allowed us to dissect the sources of fluorescence depolarization due to the dynamics of the DNA itself and the rapid reorientation of the dyes within their binding pockets. We can now exclude such depolarization, reported before from bulk spectroscopic and microscopic studies, to be due to a tilted intercalation mode: the transition moments of the YOYO chromophores are on average oriented perpendicular to the DNA helix.

The observed behavior in the overstretching transition of the DNA reveals that no dye dissociates or even reorients measurably. This is unexpected since the dsDNA into which it is intercalated is gradually converted to ssDNA, which cannot be intercalated. Furthermore, intercalated dyes would be expected to reorient if DNA they are bound to would overstretch into “S-DNA,”\textsuperscript{30} but we have shown before that YOYO does not bind to S-DNA. Most likely, our observations indicate that in the overstretching transition, only non-intercalated DNA segments undergo the elongation and changes in conformation associated with DNA overstretching. Only beyond the overstretching transition, where the DNA is held together only by isolated base pairs,\textsuperscript{42} a considerable depolarization is observed, presumably caused by an average tilting of the DNA base pairs. These unique observations on DNA dynamics could only be made thanks to our combined polarized fluorescence and DNA manipulation assay.

SUPPLEMENTARY MATERIAL

See supplementary material for further descriptions of Monte Carlo simulations, the effects of dye mobility by DNA
dynamics ("slow wobble") and of dye mobility on fluorescence time scale ("fast wobble"), transmission and high-NA corrections, and a figure of the experimental setup.

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