Supplementary Materials for

**Single-molecule polarization microscopy of DNA intercalators sheds light on the structure of S-DNA**

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Fig. S1. Intercalator tilting is prevented when dye coverage is high. (A) Force-extension curves of dsDNA in the presence of 1 µM YO-PRO-1. (B) Corresponding emission polarizations plotted as a function of DNA extension. Unpolarized illumination is used. (C) Representative fluorescence polarization micrographs of DNA extended to 1.4, 1.65, and 1.9 extension (top to bottom respectively) in the presence of 1 µM YO-PRO-1. (scale bar: 5 μm). As evidenced by the greater fluorescence signal in the y-polarized emission channel, under these conditions dyes remain nearly perpendicular to the DNA axis well beyond the overstretching transition. Note that measurements were taken using a high-salt buffer and with the fast equilibrating dye YO-PRO-1 (as opposed to SYTOX Orange or YOYO-1), since these conditions ensure successful HS-DNA generation (16).
Fig. S2. Summary of different modes of rotational motion. An intercalated dipole has a fixed mean tilt angle $\theta$ with respect to the DNA-axis. However, the dipole rapidly wobbles about its mean orientation $\{\theta, \phi\}$ within the cone prescribed by $\alpha$. Furthermore, the dipole may twirl to different azimuthal orientations $\phi$ with respect to the DNA-axis, while maintaining a constant tilt angle $\theta$. Since twirling is assumed to occur on a timescale much slower than probe wobble, we assume that on the timescale of the fluorescence lifetime (~3 ns), the mean orientation $\{\theta, \phi\}$ is constant. However, on the timescale of the camera exposure time (1 sec), each azimuthal orientation $\phi$ is visited with equal frequency.

Fig. S3. Definitions of coordinate systems and reference frames. (A) Dipole reference frame: The $z''$-axis is aligned with the mean orientation of the dipole, as it wobbles within a cone defined by $\alpha$. In this reference frame, the instantaneous orientation of the emitter is specified in the spherical coordinates $\{\chi, \xi\}$. (B) DNA reference frame: The $z'$-axis is coaligned with the DNA-axis. We assume all emitters to be tilted away from the DNA-axis by the mean polar angle $\theta$. Individual emitters are uniformly oriented at different azimuthal angles $\phi$, and can freely twirl about the DNA-axis due to Brownian twisting. (C) Experimental reference frame: The DNA lies in the $(x/y)$ image plane, and is rotated by an angle $\omega$ away from the $x$-axis. In this reference frame, dipoles have instantaneous orientations specified in spherical coordinates $\{\theta, \phi\}$.
Fig. S4. The effect of wobble and tilt upon emission polarization. (A) \( yP \) versus \( \omega \), varying \( \alpha \), is \( \theta \) fixed. (B) \( yP \) versus \( \omega \), varying \( \theta \), \( \alpha \) is fixed. For all plots, \( xP \) may be inferred from \( yP \) by vertically reflecting the curves about \( \omega = 45^\circ \), and multiplying by -1.

Fig. S5. Three experimental configurations for measuring intercalated dye orientations. (A) Experimental configuration (1): The DNA-axis is held fixed, while the excitation polarization is rotated within the image plane by an angle \( \psi \). Total fluorescence intensity \( I \psi \) is measured. (B) Experimental configuration (2): The same excitation approach is used, but the fluorescence emission is resolved into x/y-polarized components using a polarizing beamsplitter. Fluorescence polarization \( P \psi \). (C) Experimental configuration (3): The excitation polarization is fixed along the y-axis, while the DNA is rotated within the image plane by an angle \( \omega \). This was the experimental approach utilized for measurements reported in Fig. 2 of the main text.
Fig. S6. Simulated measurements corresponding to parameter sets \{\theta = 85^\circ, \alpha = 85^\circ\} and \{\theta = 53.6^\circ, \alpha = 39.1^\circ\}. (A) Using experimental configuration (1), both parameter sets yield nearly identical fluorescence intensity. (B) Using experimental configuration (2), both parameter sets yield nearly identical emission polarization. (C) Using experimental configuration (3), the parameter set \{\theta = 53.6^\circ, \alpha = 39.1^\circ\} yields a negative fluorescence polarization of appreciable magnitude, when the DNA is rotated \omega \sim 45^\circ. \_P may be inferred from \_P by vertically reflecting the curves about \omega = 45^\circ, and multiplying by -1.
Fig. S7. Intuitive description of the differences between experimental configurations. (Assuming parameters \( \theta = 53.6^\circ, \alpha = 39.1^\circ \).) (A) The DNA-axis remains fixed, and the excitation polarization is rotated, corresponding to experimental configurations (1) and (2). If the excitation polarization is oriented along the x- or y-axis, all dipoles are weakly excited, yielding an unpolarized fluorescence signal. (In the case of \( \psi = 0^\circ \), the subset of dipoles oriented along the x-axis will be severely tilted out of the image plane, leading to weak excitation, and poor fluorescence collection efficiency.) In the case of \( \psi = 45^\circ \), a subset of dipoles of similar orientations is efficiently excited, but this subset is not aligned with either the x- or y-axis along which polarization is resolved. (B) The excitation polarization remains fixed, and the DNA is rotated. As in the above case, if the DNA is oriented at \( \omega = 0^\circ \) or \( \omega = 90^\circ \), all dipoles are weakly excited, yielding unpolarized fluorescence signal. However, in the case of \( \omega = 45^\circ \), a subset of dipoles is efficiently excited that aligns closely with the y-axis of the experimental system, enabling a polarized fluorescence signal to be detected. (If, instead, the excitation polarization were oriented along the x-axis, the emission polarization would be positive.)
Fig. S8. Example ROI used for ensemble fluorescence polarization measurements. In these representative images, a DNA molecule is stretched to an extension within the overstretching transition (regime 2), and y-polarized excitation is employed. (A) Representative ROI used in the y-polarized emission channel is outlined in green. (B) Corresponding ROI used for the x-polarized emission channel. Note that ROIs primarily contain regions of densely labeled DNA.

Table S1. Accounting for energy transfer. Estimated parameters \( \{\theta, \alpha\} \) for different choices of \( \beta \), the angle between absorption and emission dipoles.

| Relative DNA extension: Within the OST (regime 2) | \( \theta \) | \( \alpha \) |
|--------------------------------------------------|----------------|------------------|
| \( \beta = 10^\circ \)                          | 85.4° ± 3.9°   | 28.3° ± 4.7°     |
| \( \beta = 20^\circ \)                          | 85.3° ± 3.6°   | 25.6° ± 5.2°     |
| \( \beta = 30^\circ \)                          | 85.2° ± 3.2°   | 20.3° ± 6.8°     |

| Relative DNA extension: Beyond the OST (regime 3) | \( \theta \) | \( \alpha \) |
|--------------------------------------------------|----------------|------------------|
| \( \beta = 10^\circ \)                          | 53.7° ± 1.4°   | 38.7° ± 1.9°     |
| \( \beta = 20^\circ \)                          | 54.0° ± 1.4°   | 37.4° ± 2.0°     |
| \( \beta = 30^\circ \)                          | 54.5° ± 1.4°   | 35.0° ± 2.2°     |
Section S1. Intercalators prevent peeling and bubble formation under low-salt conditions

Under low salt conditions, duplex DNA will typically unpeel during overstretching, whereas under high salt conditions overstretching will result in S-DNA. However, there is a subtle, but important distinction between experiments conducted under low and high intercalator coverage. When there is a high intercalator coverage, unpeeling is prohibited, even at low salt. This is because stacking of the intercalator to both bases of a base-pair stabilizes the duplex by lowering the propensity for those bases to unpair and for the two strands to separate, which, in turn, favours the flanking regions of DNA to adopt the S-form rather than the unpeeled state (15). This stabilizing effect of intercalators is reduced at low intercalator coverage (such as in our single-intercalator analyses) where unpeeling of the two strands is observed under low salt conditions, as expected. Under high salt conditions, on the other hand, S-DNA is favoured over unpeeled DNA. For this reason, we had to perform all low density, single-intercalator experiments under high-salt conditions that favour S-DNA formation.

Section S2. Relating the measured intercalator orientation to DNA structure

It is important to determine if intercalators perturb the structure of DNA adjacent to intercalated (I-)DNA, and if so, to what degree. Based on the observations summarized below, we conclude that while intercalators can influence the relative ratios of B-DNA, S-DNA and (hyperstretched) HS-DNA, they can be used to estimate the base-pair angle of non-intercalated DNA regions flanking the intercalated sites.

Polarization angle is a reporter of orientation of flanking DNA sections

Upon binding, intercalators stack between adjacent base pairs of DNA, thus lengthening the DNA. This stacking strongly enhances intercalator fluorescence by suppressing intramolecular motions and any associated non-radiative decay. Because stacking energies are maximized in a parallel configuration (43), we argue that it is highly likely that the orientation of intercalated
dyes serves as a robust reporter for the orientation of neighbouring base-pairs. This feature is further supported by NMR studies of (relaxed) DNA intercalated with YOYO-1 (44).

**I-DNA is non-tilted when S-DNA is absent**

At forces < 65 pN (regime 1), dsDNA exists in a B-DNA state for which intercalators have a very high affinity (15). Upon binding to dsDNA, the intercalator locally lengthens the backbone by 100%, but the dye is orientated approximately perpendicular to the DNA backbone (24, 44). This angle is nearly identical to that of the base-pairs in B-DNA which flank the intercalated sites. In contrast, at very high forces (>> 65 pN) and very high dye concentrations, intercalation can induce the hyperstretched (HS) state of DNA (16), instead of S-DNA. In HS-DNA, the backbone is globally lengthened by 100% compared to B-DNA. Under these conditions (measurements taken using 1μM concentration of the mono-intercalator YO-PRO-1, fig. S1), we measure negative dye polarizations of large magnitudes. This indicates that intercalators are also predominantly oriented perpendicular to the DNA-axis when flanked by HS-DNA. From these two facts, we infer that intercalated dyes prefer to adopt a perpendicular orientation to the DNA backbone.

**I-DNA is tilted only when S-DNA dominates**

Intercalator binding is drastically reduced when DNA is in the S-DNA state, compared with the B-DNA or HS-DNA states (9, 16). Furthermore, in the presence of increasing intercalator concentration, the onset of S-DNA formation has been shown to be increasingly shifted to higher forces, until – at a critical dye concentration – the transition to S-DNA is completely bypassed in favour of HS-DNA formation (fig. S1). This indicates that there is an energy penalty for intercalator binding to S-DNA. In the current work, we observe a significant tilting of bound intercalators only under conditions where nearly all of the B-DNA has been converted into S-DNA. Importantly, the tilting of the dyes displays two important characteristics: a) the tilting of
single dyes occurs in an abrupt manner, and b) under conditions in which tilting is observed, the measured angle is largely independent of the amount of intercalator binding and other changes in experimental conditions.

**I-DNA reflects the neighbouring DNA structure**

Based on the above observations, we conclude that intercalators do not induce a tilt in base-pair angle by themselves; rather, the tilt observed for intercalators reflects the structure of the neighbouring DNA domains. As a result, it follows that S-DNA possesses a tilted base-pair conformation, in contrast to B-DNA and HS-DNA which exhibit a non-tilted structure. Furthermore, the abrupt transition from a non-tilted to a tilted intercalator orientation (near the end of the overstretching transition) suggests that the tilted DNA state possesses an ordered structure which is in line with the cooperativity of the B-DNA to S-DNA transition. Finally, the fact that we observe a similar tilt angle at both very low and relatively high intercalator coverage (Figs 2 and 3 respectively, main text), makes it further unlikely that intercalation strongly alters the structure of neighbouring base pairs. Taken together, our results are most readily explained by the fact that the observed tilting angle of bound dyes reflects the intrinsic structure of flanking S-DNA domains. Note that, although intercalators can be flanked by S-DNA, their much higher affinity for both B- and HS-DNA relative to S-DNA suggests that intercalation is energetically more favourable for a perpendicular dye orientation. As a consequence, intercalators do have a notable effect on the B-S transition. Specifically, a high intercalator coverage stabilizes the B-form state, resulting in a higher onset of the overstretching transition. However, this does not affect our ability to determine the orientation of base-pairs within each state of DNA.

**Section S3. Theoretical model: Tilt wobble and twirl**

Here, we develop a mathematical framework to estimate the mean intercalator tilt angle $\theta$ and wobble cone half-aperture angle $\alpha$ from emission polarization data. Under the assumptions described below, our model applies both to the case of a single dipole twirling about the DNA-
axis, as well as an ensemble of dipoles oriented about the DNA-axis in cylindrically symmetric fashion.

Historically, time-resolved fluorescence polarization anisotropy methods were employed in the study of lipid membranes to estimate the wobble cone $\alpha$ of fluorescent probes in isotropic environments \((45, 46)\). Theoretical analysis of cylindrically-symmetric systems \((33, 47)\) such as motor protein dynamics along actin filaments \((34, 48, 49)\) led to the development of steady-state polarization imaging techniques that could distinguish probe wobble from axial tilting with respect to the symmetry axis. The model presented here is nearly identical to that of Irving et al. \((33)\). However, we employ a different mathematical approach \((50)\) that uses linear algebra to effect the numerous coordinate rotations required for our calculations. In addition to the premise of cylindrical symmetry about the DNA-axis, we make the following three assumptions:

1. We assume that individual chromophores wobble within their binding sites on a timescale much faster than their fluorescence lifetimes \((24)\). In the time that elapses between the absorption and emission of a photon, a chromophore explores the entire cone $\alpha$ to which it is rotationally confined. Under these circumstances, the excitation polarization will not bias the orientation within the cone $\alpha$ at which light is emitted.

2. We assume that intercalator twirling about the DNA-axis occurs on a timescale much slower than the fluorescence lifetime \((31)\) (yet still much faster than the exposure time of our camera). In the period that elapses between absorption and emission, the intercalator will not have sufficient time to twirl to a new mean orientation with respect to the DNA (even though it explores the complete wobble cone defined by $\alpha$). Consequently, the excitation polarization will influence the polarization of emitted light.

3. Energy transfer between the YOYO-1 chromophores may introduce a fixed angle between the absorption and emission transition dipole moment \((30, 51)\). For simplicity, we initially develop a model that assumes the absorption and emission axes are parallel. In following sections, the model is modified to account for distinct absorption and emission transition dipole moments, and we re-analyze polarization data using this additional parameter.

The different modes of rotational motion considered are summarized in fig. S2. To aid our calculations, we will use three different reference frames shown in fig. S3. Transformations
between reference frames are accomplished by a series of coordinate rotations, as explained in the following sections.

**Preliminary considerations**

To begin constructing our theoretical framework, we initially consider a rotationally immobilized dipole emitter. We use the unit vector \( \hat{\mu}(\theta, \phi) = [\sin(\theta)\cos(\phi), \sin(\theta)\sin(\phi), \cos(\theta)]^T \) to specify the orientation of the emitter within the experimental reference frame. We denote the electric fields that would be incident at the microscope image plane associated with dipoles aligned along the x-, y-, and z-axes as: \( u_{x,y}^{x,y,z}(r) \). Superscripts refer to the orientation of the emitter, subscripts refer to the polarization of the electric field. \( r \) is a two-dimensional vector specifying a location within the image plane. In principle, a z-polarized electric field is also produced at the microscope image plane. However, owing to the long focal length of the microscope tube-lens, image plane fields are approximated as paraxial, and have negligible z-component. To calculate the image plane fields \( u_{x,y}^{x,y,z}(r) \), one must incorporate the high numerical aperture of the microscope objective, and the immersion media (in our case: 1.2 NA, water immersion). Many previous works (18, 52-55) address that calculation, and we do not repeat the details here. The x-/y-polarized image plane electric fields \( u_{x,y}^{x,y,z}(r) \) associated with an emitter of arbitrary orientation \( \hat{\mu}(\theta, \phi) \) are given by

\[
\begin{align*}
    u_x^\mu(r) &= \hat{\mu}^T(\theta, \phi)u_x(r); \\
    u_y^\mu(r) &= \hat{\mu}^T(\theta, \phi)u_y(r)
\end{align*}
\]

(S4)

Where: \( u_{x,y}(r) = [u_x(r) \quad u_y(r) \quad u_z(r)]^T \). The total x-/y-polarized intensities \( I_{x,y}^\mu \) incident at the microscope image plane for a dipole of orientation \( \hat{\mu}(\theta, \phi) \) are then computed as
\[ I_x^\mu = \int_R |u_x^\mu (r)|^2 dr; \quad I_y^\mu = \int_R |u_y^\mu (r)|^2 dr \quad (S5) \]

Where a two-dimensional integration is performed over a region \( R \) of the image plane. This integral is computed numerically using the image plane electric fields \( u_{x,y}(r) \). This operation corresponds to summing the pixel intensities within the respective x/y-polarization channels recorded on a camera.

For a rotating dipole emitting light at different orientations over the course of a single camera exposure, the polarized intensity at the image plane is

\[
I_x^\eta = \oint \oint \eta(\theta, \varphi) (u_x^\dagger (r) \hat{\mathbf{\mu}}(\theta, \varphi) \hat{\mathbf{\mu}}^\dagger (\theta, \varphi) u_x (r)) \sin(\theta) d\theta d\varphi dr \\
I_y^\eta = \oint \oint \eta(\theta, \varphi) (u_y^\dagger (r) \hat{\mathbf{\mu}}(\theta, \varphi) \hat{\mathbf{\mu}}^\dagger (\theta, \varphi) u_y (r)) \sin(\theta) d\theta d\varphi dr \quad (S6)
\]

Where \( \eta(\theta, \varphi) \) is a weighting function that specifies the relative frequency with which the dipole emits at different orientations \( (41) \). Taking the terms \( u_{x,y}(r) \) and \( u_{x,y}^\dagger (r) \) outside of the integral over the unit sphere, we re-write Equations (S3) as

\[
I_x^\eta = \oint \oint u_x^\dagger (r) M^\eta u_x (r) dr \\
I_y^\eta = \oint \oint u_y^\dagger (r) M^\eta u_y (r) dr \quad (S7)
\]

Where the 3x3 matrix \( M^\eta \) is given by the formula

\[
M^\eta = \oint \oint \eta(\theta, \varphi) (\hat{\mathbf{\mu}}(\theta, \varphi) \hat{\mathbf{\mu}}^\dagger (\theta, \varphi)) \sin(\theta) d\theta d\varphi \quad (S8)
\]
Hence, to calculate the polarized emission intensities $I_{xy}^\eta$, we must first determine the matrix $M^\eta$, then evaluate the integrals in Equations (S4). Instead of directly attacking the integral in Equation (S5), our strategy will be to calculate an intermediate $M^\eta$ in each of the reference frames detailed in fig. S3. By successively transforming coordinate systems, we can derive a suitable $M^\eta$ for the desired experimental reference frame.

The diagonal entries of $M^\eta$ are the second moments of the emitting dipoles’ projections onto the $x$-, $y$-, and $z$-axis of the experimental reference frame. Instead of using Equations (S4) to compute the intensities $I_{xy}^\eta$, one may equivalently use an approach described in (52)

$$
I_x^\eta = K_1M^\eta(1,1) + K_2M^\eta(2,2) + K_3M^\eta(3,3) \\
I_y^\eta = K_2M^\eta(1,1) + K_1M^\eta(2,2) + K_3M^\eta(3,3)
$$

(S9)

In Equations (S6), parentheses refer to specific diagonal entries of $M^\eta$, and the scalar quantities $K_{1,2,3}$ are calculated from the collection angle of the objective lens (64.5° for a water immersion objective, 1.2 NA). For our experimental system: $\{K_1 = 0.86, K_2 = 0.15, K_3 = 0.26\}$. Either Equations (S4) or (S6) will correctly account for effects associated with emitting dipoles tilted out of the image plane, as well as depolarization due to the high numerical aperture objective lens.

**Fast wobbling within a cone**

We use the dipole-aligned coordinate system of fig. S3A to model emitter wobble within the cone $\alpha$. For this calculation, we assume the emitter changes orientation on a timescale much faster than the fluorescence lifetime. The weighting function $\eta^\text{cone}(\chi, \zeta)$ associated with fast wobble within a cone will not be affected by the excitation polarization. Assuming the wobbling dipole visits each orientation within the cone $\alpha$ with equal frequency
\[ \eta_{\text{cone}}(\chi, \xi) = \begin{cases} \frac{1}{2\pi(1-\cos(\alpha))} & \text{if } \mu^\dagger(\chi, \xi) \mu(0,0) > \cos(\alpha) \\ 0 & \text{Otherwise} \end{cases} \] (S10)

We evaluate the integral

\[ M_{\text{cone}}(\alpha) = \int_0^{2\pi} \int_0^\alpha \eta_{\text{cone}}(\chi, \xi) (\hat{\mu}(\chi, \xi) \hat{\mu}^\dagger(\chi, \xi)) \sin(\chi) d\chi d\xi \]

\[ = \begin{bmatrix} 0 & 0 & 0 \\ 6 & \frac{(1-\cos(\alpha))(\cos(\alpha)+2)}{6} & 0 \\ 0 & 0 & \frac{(\cos^3(\alpha)-1)}{3(\cos(\alpha)-3)} \end{bmatrix} \] (S11)

Having computed \( M_{\text{cone}}(\alpha) \) in the dipole reference frame, it is straightforward to transform this quantity to the DNA reference frame using two coordinate rotations

\[ M_{\text{cone}}(\alpha, \theta, \phi) = R_z(\phi) R_y(\theta) M_{\text{cone}}(\alpha) R_y^\dagger(\phi) R_z^\dagger(\theta) \] (S12)

Where \( R_z(\phi) \) denotes a rotation about the \( z'' \)-axis by the angle \( \phi \) and \( R_y(\theta) \) denotes a rotation about the \( y'' \)-axis by the angle \( \theta \)

\[ R_z(\phi) = \begin{bmatrix} \cos(\phi) & -\sin(\phi) & 0 \\ \sin(\phi) & \cos(\phi) & 0 \\ 0 & 0 & 1 \end{bmatrix}; \quad R_y(\theta) = \begin{bmatrix} \cos(\theta) & 0 & \sin(\theta) \\ 0 & 1 & 0 \\ -\sin(\theta) & 0 & \cos(\theta) \end{bmatrix} \] (S13)
**Slow twirling about the DNA-axis**

Using the result for $M^{cone}(\alpha, \theta, \phi)$ from the previous section, we now turn our attention to deriving an appropriate matrix $M^{DNA}(\alpha, \theta)$ for a dipole twirling about the DNA-axis at a rate much slower than the fluorescence lifetime, yet much faster than the camera exposure time. Note that this case is identical to an ensemble of dipoles oriented about the DNA-axis in cylindrically symmetric manner. Under these circumstances, the excitation polarization will preferentially excite certain dipole orientations, and will therefore play a crucial role determining $M^{DNA}(\alpha, \theta)$.

The amount of optical energy absorbed, and hence the amount of light emitted, by a single dipole is proportional to the cosine-square of the angle between the dipole moment and the excitation polarization. For a single dipole wobbling within a cone $\alpha$ centered about the mean orientation $\{\theta, \phi\}$ in the DNA reference frame, the energy absorbed, $U_{abs}(\alpha, \theta, \phi, \omega)$, is calculated as

$$U_{abs}(\alpha, \theta, \phi, \omega) = A \left( E^{\dagger}(\omega) M^{cone}(\alpha, \theta, \phi) E(\omega) \right)$$  \hspace{1cm} (S14)

In Equation (S11), $E(\omega)$ is a vector specifying the axis of the excitation polarization. The constant $A$ is the absorption efficiency of the dipole. In the experimental reference frame, the DNA is rotated about the $z$-axis by the angle $\omega$, while the excitation polarization is held constant. However, in the DNA reference frame, the DNA molecule remains aligned along the $z'$-axis, as the excitation polarization is rotated about the $x'$-axis within the $y'/z'$-plane

$$E(\omega) = \begin{bmatrix} 0 \\ \cos(\omega) \\ \sin(\omega) \end{bmatrix}$$ \hspace{1cm} (S15)
The scalar $E$ is a constant to account for the amplitude of the excitation field. $M^{DNA}(\alpha, \theta, \omega)$ is an integral of the individual $M^{cone}(\alpha, \theta, \phi)$ at each azimuthal orientation $\phi$, weighted by $U_{\text{abs}}(\alpha, \theta, \phi, \omega)$

$$
M^{DNA}(\alpha, \theta, \omega) = \frac{1}{2\pi} \int_0^{2\pi} A\left(\mathbf{E}^\dagger(\omega)M^{cone}(\alpha, \theta, \phi)\mathbf{E}(\omega)\right)M^{cone}(\alpha, \theta, \phi) d\phi 
$$

The resulting integral may be performed analytically, but is tedious. In practice, we evaluated Equation (S13) using the Symbolic Math Toolbox available in the software package MATLAB.

**Transformation to the experimental reference frame**

Having determined $M^{DNA}(\alpha, \theta, \omega)$ in the DNA reference frame, the final step is to perform a coordinate rotation into the experimental reference frame. This is accomplished by rotating 90$^\circ$ about the $y'$-axis, then rotating by the angle $\omega$ about the $z'$-axis

$$
M^{Exp}(\alpha, \theta, \omega) = R_z(\omega)R_y(90^\circ)M^{DNA}(\alpha, \theta, \omega)R_y(90^\circ)R_z^\dagger(\omega)
$$

The appropriate rotation matrices are

$$
R_z(\omega) = \begin{bmatrix}
\cos(\omega) & -\sin(\omega) & 0 \\
\sin(\omega) & \cos(\omega) & 0 \\
0 & 0 & 1
\end{bmatrix}; \quad R_y(\theta) = \begin{bmatrix}
0 & 0 & 1 \\
0 & 1 & 0 \\
-1 & 0 & 0
\end{bmatrix}
$$

$M^{Exp}(\alpha, \theta, \omega)$ is substituted for $M^\theta$ in Equations (S4), and the relative intensities of $I^{(\alpha, \theta, \omega)}_x$ and $I^{(\alpha, \theta, \omega)}_y$ are calculated. For clarity, we have replaced the general $\eta$ as superscript with our specific model parameters $\{\alpha, \theta, \omega\}$. In our derivation, we have implicitly chosen the excitation polarization $\mathbf{E}$ to lie along the $y$-axis in the experimental reference frame. To determine $I^{(\alpha, \theta, \omega)}_{x,y}$
for x-polarized illumination (i.e., $I_x$ and $I_y$ as opposed to $I_x$ and $I_y$ using the prefix notation from the main text), simply compute $M^{Exp.}(\alpha, \theta, 90^\circ - \omega)$, and swap the resulting values of $I_x^{(\alpha, \theta, 90^\circ - \omega)}$ and $I_y^{(\alpha, \theta, 90^\circ - \omega)}$. Using this model, the theoretically expected emission polarizations $xP$ and $yP$ can be determined using Equations (1) (main text) for any choice of parameters \{\alpha, \theta, \omega\}.

Section S4. Distinguishing wobble and tilt

Using the theoretical model developed in the preceding sections, we examine how changes in \theta and \alpha affect the predicted emission polarizations. As a rule of thumb, the overall shapes of the $xP$ (or $yP$) versus \omega plots reveal \theta, while the amplitudes of $xP$ and $yP$ reveal \alpha. In the bulk measurements presented in the main text, we used nonlinear least squares fitting to estimate \{\theta = 53.6^\circ, \alpha = 39.1^\circ\} for intercalators bound to DNA extended beyond the overstretching transition.

In fig. S4A, we fix $\theta = 53.6^\circ$, and plot $xP$ as a function of DNA orientation $\omega$ for different values of \alpha. The magnitude of the expected polarization decreases with increasing \alpha. A larger wobble-cone will cause fluorescence emission to become increasingly depolarized, regardless of the DNA orientation $\omega$ and the tilt $\theta$. However, the maximum polarization amplitude is achieved near $\omega \sim 45^\circ$ for all values of \alpha considered. In fig. S4B, we fix $\alpha = 39.1^\circ$ and vary the tilt parameter $\theta$.

For different tilt angles, the $yP$ plots change markedly. As $\theta$ decreases, the emission polarization becomes positive for $\omega \sim 0^\circ$, and negative for $\omega \sim 90^\circ$. This is to be expected, since the intercalators will be closely aligned with the DNA-axis as $\theta$ approaches zero.
**Section S5. Why is it necessary to rotate the DNA?**

To accurately discern the mean tilt angle $\theta$ of intercalated dyes, we rotate an optically trapped DNA molecule by an angle $\omega$ within the image plane of our fluorescence microscope. Dyes are alternately excited using x/y-polarized illumination, and fluorescence is simultaneously resolved into x/y-polarized imaging channels using a fixed polarizing beamsplitter. It is reasonable to ask: *Could similar measurements be obtained by keeping the DNA orientation fixed, and rotating the excitation polarization instead of the DNA?* Excitation-polarization rotation is a technique that has been utilized effectively by many authors in the past to determine in-plane absorption dipole orientations (23, 25-27, 56). Continuous rotation of the excitation polarization is readily accomplished using an electro-optic modulator, and is admittedly a much simpler experimental approach than manual realignment of the DNA orientation. However, after initially attempting an experimental approach based on excitation polarization rotation, we concluded that this technique would not permit us to unambiguously determine the intercalator tilt angles. In this section, we present simulation results illustrating the strengths and drawbacks of different proposed experimental approaches. From these calculations, we confirm that rotating the DNA molecule within the microscope image plane is the most expedient means of experimentally observing tilted dipole orientations.

In this section, we compare three unique experimental approaches shown schematically in fig. S5.

We summarize each approach as follows:

1. The DNA orientation is fixed at $\omega = 0^\circ$, and the excitation polarization is rotated within the image plane by an angle $\psi$. In this experimental configuration, we measure only the total emitted fluorescence intensity $\Psi I$, without resolving fluorescence into x/y-polarized components using a polarizing beamsplitter.

2. The DNA orientation is fixed, and the excitation polarization is rotated. However, in this scenario, we resolve fluorescence emission into x/y-polarized components, and compute emission polarization ratios $\Psi P$ as a function of the excitation polarization orientation $\psi$. 
Finally, we consider the experimental approach ultimately used in Fig. 2 of the main text. The DNA orientation is rotated, while the excitation polarization is kept fixed along either the x- or y-axis, and the emission polarization ratio $\gamma P$ is measured as a function of $\omega$.

To analyze each of these three experimental configurations, we utilized the same theoretical framework presented in Section S1. Briefly, in configurations (1) and (2) discussed above, instead of using rotation matrices to simulate rotation of the DNA about the z-axis of the experimental reference frame, we fix the DNA along the x-axis, and rotate the excitation polarization $E$ by an angle $\psi$ about the z-axis. We calculate the expected intensity and polarization measurements that would result under two sets of conditions: (1) We set the mean tilt and cone angle to $\{\theta = 85^\circ, \alpha = 85^\circ\}$. Under these circumstances, the dipoles are highly rotationally mobile, and explore nearly a complete hemisphere of orientations. (2) Next, we consider the parameters estimated from the measurements reported in Fig. 2 of the main text: $\{\theta = 53.6^\circ, \alpha = 39.1^\circ\}$. For each set of $\{\theta, \alpha\}$ simulated measurements are plotted in fig. S6.

Experimental configurations (1) and (2) exhibit slight changes in overall fluorescence intensity $\psi I$ and fluorescence polarization $\psi P$. Both of the two cases $\{\theta = 85^\circ, \alpha = 85^\circ\}$ and $\{\theta = 53.6^\circ, \alpha = 39.1^\circ\}$ yield nearly identical measurements, rendering it unlikely that these sets of parameters can be experimentally distinguished (considering that the precision of individual polarization measurements is estimated as $\sigma_P = 0.16$). However, using experimental configuration (3), the magnitude of the fluorescence polarization $\gamma P$ changes markedly as the DNA is rotated through $\omega = 45^\circ$ for the case $\{\theta = 53.6^\circ, \alpha = 39.1^\circ\}$. In contrast, for the case $\{\theta = 85^\circ, \alpha = 85^\circ\}$, the expected polarization remains nearly flat for all DNA orientations $\omega$. From these calculations, we concluded that rotating the DNA orientation $\omega$ provided the most effective means of distinguishing intercalator wobble from tilt.

In fig. S7 we intuitively illustrate the key differences between experimental configurations (1), (2), and (3), using the underlying parameters $\{\theta = 53.6^\circ, \alpha = 39.1^\circ\}$ are valid: By keeping the
DNA orientation fixed, and rotating the excitation polarization, different subsets of dipoles are preferentially excited. However, no subset of excited dipoles will closely align with the x/y-axes along which the polarization is resolved. Hence, the fluorescence emission will appear unpolarized for all choices of excitation polarization $\psi$. Alternatively, using experimental configuration (3), as the DNA is rotated to intermediate orientations $\omega \sim 45^\circ$, the subset of excited dipoles will align closely with the y-axis of the experimental system. This configuration will yield a negative polarization signal of appreciable magnitude, permitting confirmation of tilted dipoles with respect to the DNA axis.

To conclude this section, we note that if one wished to record measurements identical to those of experimental configuration (3), without manually reorienting the DNA molecule, a procedure could, in principle, be devised: Instead of rotating the DNA-axis, it would be necessary to simultaneously rotate both the excitation polarization, and the axes along which the emission polarization is resolved. Such an approach could be implemented by mounting a polarizing beamsplitter on a rotation stage within the emission pathway of the fluorescence imaging system. Such an approach would be more complex experimentally. Hence, we favored rotating the DNA molecule via configuration (3).

**Section S6. The effect of energy transfer upon emission polarization**

We extend our theoretical model to account for the possibility of energy transfer between the individual YOYO-1 chromophores. The same adjustment to our theoretical model may be used to estimate the effects of energy transfer between separate dye pairs (51), or a separation angle between absorption and emission dipole moments of the same chromophore (30). Following our previous work (24), we assume that both YOYO-1 chromophores are tilted with respect to the DNA axis by an identical angle $\theta$, but are rotated from each other by an azimuthal angle $\beta$ about
the DNA-axis. \( U_{abs}(\alpha, \theta, \phi, \omega) \) is calculated as before using Equation (S11). However, we modify Equation (S13) to compute the following weighted integral

\[
M^{DNA}(\alpha, \theta, \omega) = \frac{1}{2\pi} \int_{0}^{2\pi} A(E^\prime(\omega) M^{cone}(\alpha, \theta, \phi) E(\omega)) M^{cone}(\alpha, \theta, \phi + \beta) d\phi
\]

Effectively, light is absorbed into the cone defined by the matrix \( M^{cone}(\alpha, \theta, \phi) \), and emitted by the cone defined by the matrix \( M^{cone}(\alpha, \theta, \phi + \beta) \). To examine how energy transfer may affect our estimates for the fitted parameters \( \{\theta, \alpha\} \) we chose different values of \( \beta \), and re-ran the analysis of fluorescence data presented in Fig. 2D and E from the main text (DNA extension within and beyond the OST at high dye coverage, regimes 2 and 3 respectively). The estimated values of \( \theta \) and \( \alpha \) are given in table S1. A smaller choice of \( \beta \) results in an increased estimate of the cone \( \alpha \). In a cylindrically symmetric system, both energy transfer and fast probe wobble will cause fluorescence depolarization regardless of the orientation of the excitation polarization. Notably, for different choices of \( \beta \), the estimated tilt \( \theta \) remains remarkably consistent. This owes to the fact that \( \theta \) is primarily inferred from the DNA orientations \( \omega \) at which the emission polarization magnitude is maximized and minimized. This feature is minimally affected by isotropic depolarization processes.

Energy transfer between separate YOYO-1 dyes, and a finite angle between absorption and emission dipole moments (of the same chromophore) may also induce depolarization, affecting estimates of \( \alpha \). Either of these cases may be modeled using Equation (S16), provided that the correct choice of \( \beta \) is used. The results shown in table S1 demonstrate that energy transfer will cause larger estimated values of the cone angle parameter \( \alpha \), if the separation angle \( \beta \) is chosen to be too small (or chosen to be zero, as in the main text). Estimates of the average tilt angle \( \theta \), however, will not be strongly perturbed. To support this statement, we note that in both the cases
of single-dye measurements taken using YOYO-1 and SYTOX Orange (under these low labelling densities, energy transfer between separate dyes is expected to be negligible), estimated tilt angles \( \theta \) were nearly identical to the ensemble YOYO-1 measurements, even though smaller values of \( \alpha \) were obtained.

**Section S7. How rapidly do intercalators twirl?**

We briefly estimate the rate at which the DNA undergoes Brownian twisting, causing intercalated dipoles to twirl about the DNA-axis. Time-resolved fluorescence anisotropy measurements have characterized DNA twisting on nanosecond timescales using intercalated ethidium bromide \((57)\). Early theoretical studies \((31, 58)\) calculated the torsional rigidity constant \( C \) of DNA, which relates the torque about the DNA-axis to the amount of applied twist. Later measurements investigated the relationship between \( C \) and DNA sequence \((59)\), as well as supercoiling \((60)\).

According to the analysis of Barkley and Zimm \((31)\), the mean-square angular deviation \( \sigma^2_\theta(t) \) as a function of time \( t \) due to DNA-twisting about the molecule’s long axis is given by the formula

\[
\sigma^2_\theta(t) = k_B T L / C + 2 D t \tag{S20}
\]

\( C \) has been estimated as \( \sim 4 \times 10^{-19} \text{ erg cm} \), \( D \) is the rotational diffusion constant associated with rotation about the DNA-axis (estimated by Barkley and Zimm as \( \sim 3.17 \times 10^6 \text{ rad}^2/\text{sec} \)), \( L \) is the DNA persistence length (taken as 32.5 nm) \( T \) is room temperature and \( k_B \) is Boltzmann’s constant. After \( \sim 100 \text{ nsec} \), the DNA twists an average of \( \sim 56^\circ \) from its initial orientation. Hence, intercalator twirling occur on a timescale much slower than the fluorescence lifetime \( \sim 3 \text{ nsec} \), yet much faster than the camera frame rate \( (1 \text{ sec}) \).

**Section S8. Rapidly toggling between x-/y-polarized illumination is equivalent to unpolarized excitation within the image plane**

Toggling between x-/y-polarized illumination should be equivalent to continuous rotation of the excitation polarization within the image plane, (or circularly polarized excitation). We prove this as follows: Assume a molecule has an absorption dipole moment defined as the vector \( \mathbf{\mu}^T = [x \ y \ z] \). In the case of excitation using equal proportions of x-/y-polarized light, the total energy absorbed will be

\[
U_{abs} = \frac{1}{2} \left| \mathbf{\mu}^T \hat{x} \right| + \frac{1}{2} \left| \mathbf{\mu}^T \hat{y} \right| = \frac{1}{2} (x^2 + y^2) \quad (S21)
\]

Where \( \hat{x} \) and \( \hat{y} \) are unit vectors pointing along the x- and y-axis respectively. If the excitation polarization were instead rotated continuously, such that it spends equal time at every possible orientation, the energy absorbed will be

\[
U_{abs} = \frac{1}{2\pi} \int_0^{2\pi} \left| \mathbf{\mu}^T \begin{bmatrix} \cos(\varphi) \\ \sin(\varphi) \\ 0 \end{bmatrix} \right|^2 \, d\varphi = \frac{1}{2} (x^2 + y^2) \quad (S22)
\]

Where in the above expression we integrate uniformly over every possible excitation polarization in the image plane. Both illumination configurations yield equivalent results, and may be used interchangeably. In closing, we note that neither of these approaches yield truly unpolarized excitation, as there is negligible z-polarized contribution when using an epi-illumination geometry. TIRF illumination, or a second beam propagating along the x-axis (e. g. using light sheet) could provide z-polarized excitation.
Section S9. Because of reduced intercalator affinity for S-DNA, tilting is only observed at the end of the OST

In Fig. 4 of the main text, we found that dye tilting is only observed at the end of the OST, even though the transition from B- to S-DNA should proceed linearly during the OST. This rather unexpected behavior is explained by the fact that the binding affinity of intercalators to S-DNA is drastically lower than to B-DNA. This has been demonstrated before (9, 16) (where regions of S-DNA are associated with little or no fluorescent signal compared to bright intercalated sections of B-DNA). As a result, most I-DNA that exists during overstretching is flanked by remaining B-form DNA. Thus, even when the overstretching transition is 50% complete (i.e., where there is 50% S-DNA and 50% B-DNA), the vast majority of I-DNA will be flanked by B-DNA. As a result, there will be little change in fluorescence polarization. A significant change in fluorescence polarization will only be observed when most I-DNA is flanked by S-DNA; this will only occur near the end of the overstretching transition, where the fraction of B-DNA is <<10%. In other words, the change in fluorescence polarization is not linearly proportional to the fraction of B-DNA during overstretching. As a result, there is a relatively sharp switch from a non-tilted to a tilted conformation of I-DNA that occurs at the end of the overstretching transition when most of the B-DNA is converted to S-DNA.

Section S10. ROI selection for ensemble fluorescence polarization measurements

In this section, we provide details of how ROIs were selected to ensure that only regions of densely labeled DNA were included in polarization measurements. To construct an ROI, two ‘end-points’ were manually selected along the DNA axis, and a line segment was drawn between them. All camera pixels whose centers fell within ~195 nm of the line segment (1.5 camera pixels) were included in the ROI. Each ROI used for our measurements was manually inspected to ensure that it contained only labeled DNA (ROIs were chosen such that unlabeled/unpeeled
regions were not included for our fluorescence signal measurements). Line segments used for constructing a given ROI generally encompassed 50% – 90% of the overall stretched DNA length. The summed fluorescence intensities within these ROIs was computed, then divided by the number of pixels contained in the ROI to determine the average signal. Next, a ROI containing only fluorescence background was generated by constructing two line segments parallel to the ROI containing signal from the labeled DNA, but translated 5 camera pixels in either direction along the axis perpendicular to the DNA-axis. Average background counts were subsequently subtracted from the average signal counts. To ensure that identical ROIs were selected for both the x- and y-polarization emission channels, an affine transformation was determined for mapping pixel coordinates between the two channels. To determine an appropriate transformation matrix, images of optically-trapped microspheres were used as reference points in the two emission channels. A representative example of an ROI is shown in fig. S8.