Mixed confinement regimes during equilibrium confinement spectroscopy of DNA

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We have used a combination of fluorescence microscopy experiments and Pruned Enriched Rosenbluth Method simulations of a discrete wormlike chain model to measure the mean extension and the variance in the mean extension of λ -DNA in 100 nm deep nanochannels with widths ranging from 100 nm to 1000 nm in discrete 100 nm steps. The mean extension is only weakly affected by the channel aspect ratio. In contrast, the fluctuations of the chain extension qualitatively differ between rectangular channels and square channels with the same cross-sectional area, owing to the “mixing” of different confinement regimes in the rectangular channels. The agreement between experiment and simulation is very good, using the extension due to intercalation as the only adjustable parameter.

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I. INTRODUCTION

The thermodynamic properties of DNA confined in a nanochannel have been the subject of substantial debate¹ since experiments by Reisner *et al.*² almost a decade ago suggested a possible shortcoming in the blob theory of Daoud and de Gennes.³ Since that time, intense theoretical and simulation work on channel-confined DNA has produced a more nuanced understanding of confined semiflexible chains, but these new predictions now require experimental investigation. Confinement spectroscopy⁴ is a potentially powerful approach. This method uses a nanochannel with a fixed depth and a variable channel width, thereby probing many different channel sizes in a single device. The comparison of confinement spectroscopy data to theory implicitly assumes that the thermodynamics of a confined chain in a rectangular channel can be mapped to a square channel through the geometric mean of the channel size,^{4,5} as suggested by comparing the scaling results from Flory theories for blob-regimes in rectangular and square channels.⁶ However, when one uses confinement spectroscopy to test the crossover between different regimes of confinement, the experimental design leads to “mixing” of confinement regimes, where the channel height corresponds to a fixed confinement regime while the channel width moves between different regimes (Fig. 1). In the present contribution, we use a combination of experiment and simulation to show that such regime “mixing” only has a small effect on the extension of the chain. In contrast, regime mixing leads to qualitatively different fluctuations in the extension for chains in square and rectangular channels of equal cross-sectional area.

There is now a relative consensus that four regimes characterize the thermodynamics of DNA confined in a

nanochannel as a function of the channel size D , as suggested first by Odijk.⁶ In the strongest confinement, known as the Odijk regime,⁷ the chain is renormalized into a series of deflection segments. In this regime, hard-core repulsion exists between the chain and the channel walls but the chain stiffness prevents intrachain excluded volume interactions. From a theoretical perspective, the Odijk regime for nanochannel confinement is very well established.^{8,9} As the channel size increases further, the chain is able to backfold and enters a poorly understood “transition” regime. Provided the chain is sufficiently long,^{6,10–12} numerous simulations of confined wormlike chains indicate that (i) the average chain extension scales^{10,12–15} like $X \sim D^{-1}$ and (ii) this regime ends when $D \approx 2l_p$, with l_p being the persistence length of the chain.^{6,12,15} As the channel size increases even further, the chain enters into a so-called “extended de Gennes” regime¹² where the chain consists of sausage-like, anisometric blobs.^{6,16} At the largest values of D , the weak confinement results in the classical de Gennes regime,³ where the chain consists of isometric blobs. These two blob regimes were given similar names¹² to highlight that the scaling for the extension in the isometric blob de Gennes regime, $X \sim D^{-2/3}$, “extends” into the anisometric blob regime. The latter scaling is based on a Flory exponent $\nu = 3/5$; the more accurate estimate $X \sim D^{(\nu-1)/\nu} \sim D^{-0.7015}$ is in excellent agreement with simulation data for long chains in the extended de Gennes and de Gennes regimes.^{12–15,17}

It is important to recognize that, while the extension of the confined chain is the simplest observable metric and quite relevant for genomic applications of confined DNA,^{18–24} this single thermodynamic variable is likely insufficient to resolve many of the open questions surrounding different regimes. Rather, we require measurements of both the average chain extension and its variance, the latter being related to the effective spring constant of the chain. For example, the scaling

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FIG. 1. Schematic illustration of mixing of confinement regimes in confinement spectroscopy. R corresponds to a rectangular channel with depth $D_1 = 100$ nm and width $D_2 = 400$ nm. S shows an equivalent square channel of dimension $D_{Av} = 200$ nm. The purple face indicates the projection of the DNA onto the wall of height D_1 , which lies in the transition regime. The yellow face indicates the projection of the DNA onto the wall of height D_{Av} which lies in the extended de Gennes regime. The green face indicates the projection of the DNA onto the wall of height D_2 which lies in the de Gennes regime. The black curves represent polymer chains and red circles represent blobs.

for the chain extension with channel size cannot distinguish between the extended de Gennes and de Gennes regimes.¹² However, the fluctuations of the chain should scale differently with channel size in these two regimes,¹⁷ allowing an unambiguous demarcation of the border between them. In a similar manner, the theory of the transition regime may ultimately be resolved through an analysis of the variance of the chain extension along with its mean extension.

Confinement spectroscopy is a particularly attractive approach to obtain such data, using the precision channel sizes afforded by electron-beam (e-beam) lithography and reactive ion etching while avoiding the high cost of producing many devices. The original confinement spectroscopy experiments⁴ used a fixed depth D_1 and a continuously tapered width $D_2(x)$, where x is the axial distance down the channel. In principle, one can access a wide range of average channel sizes, $D_{Av} \equiv \sqrt{D_1 D_2}$, in a single device, with the resolution limited by the step size used to pattern the taper. The result is a massive reduction in the cost and time relative to fabricating individual devices with square channels for each value of D_{Av} . However, there are two reasons to question the ability of confinement spectroscopy data to test the prevailing theories. First, while the mapping to an equivalent square channel via D_{Av} seems reasonable for small aspect ratios, confinement spectroscopy typically requires very large aspect ratios $D_2(x)/D_1$ to access multiple regimes of confinement (based on D_{Av}) at a single etch depth D_1 . Second, a continuous taper leads to a configurational entropy gradient in the channel, which can cause the DNA to gradually drift to the wider side of the taper. Trapping the DNA at a particular value of D_{Av} may require applying an opposing force, either an electric field or a fluid flow, to maintain the DNA center of mass position at a fixed location x . While this opposing force is generally weak,^{4,5} its presence implies that confinement spectroscopy in a tapered nanochannel is an inherently non-equilibrium measurement.

In the present contribution, we employ the confinement spectroscopy concept^{4,5} for equilibrium measurements, thereby removing the second caveat and allowing us to address issues related to accessing multiple regimes in a

FIG. 2. Schematic of the device for equilibrium confinement spectroscopy. Region C represents an array of nanochannel cascades of fixed depth $D_1 = 100$ nm and variable width D_2 varying from 800 nm down to 100 nm in 100 nm decrements with an additional 1000 nm wide region prior to the 800 nm region to facilitate loading of DNA with minimal shear. The nanochannels are connected to loading ports (1–4) through 50 μm wide, 1 μm deep microchannels (A and B). Region D shows two constant width regions which are 45 μm long each and connected by a 5 μm long tapered region.

confinement spectroscopy device. In our device, depicted schematically in Fig. 2, we use a fixed depth $D_1 = 100$ nm and a piecewise constant function $D_2(x)$ that cascades from a maximum channel width of 800 nm to 100 nm in 100 nm increments, with an additional 1000 nm wide region that facilitates loading and alignment between the nanochannels and microchannels during device fabrication. Using the latter region for measurement as well gives us nine values of D_2 , where $D_{Av}/l_p \in [1.40, 4.70]$ traverses the extended de Gennes and transition regimes. Each fixed value of D_2 is maintained for a distance of 45 μm , which is much longer than the extension of λ -DNA in any of these channels. We use a transient pressure-driven flow to overcome the entropic penalty to move a DNA molecule to a new channel width, and then we make our measurements under force-free conditions in the absence of a pressure gradient. Our experiments thus span from the largest aspect ratios¹⁸ used previously for channel-like confinement down to the ideal case of a square nanochannel, albeit with a data density that is much lower than the continuous taper case.^{4,5}

Since we do not have access to a set of square nanochannels with the same values of D_{Av} as our confinement spectroscopy device, we have adopted a simulation-based approach to assess the role of the aspect ratio. To this end, we use Pruned-Enriched Rosenbluth Method (PERM) simulations^{25,26} of an off-lattice, discrete wormlike chain model of confined DNA, following our recent work in this area.^{14,27,28} We first simulated the mean extension and its variance for the rectangular channels used in our experiments, using the ionic strength of the buffer to compute the effective width²⁹ and persistence length^{30,31} of the DNA. The increase of the backbone length due to the intercalating dye is thus the only free parameter. The rather good quantitative agreement between experiment and simulation thus allows us to use simulations to assess the effect of the aspect ratio through further PERM simulations in square channels of width $D = D_{Av}$.

II. EXPERIMENTAL METHODS

A. Device fabrication

The devices were fabricated on 4 in. diameter, 0.5 mm thick fused silica substrates (Hoya). The nanochannel funnels were created via electron beam lithography (Leica VB6) followed by a $\text{CF}_4:\text{CHF}_3$ reactive ion etching (RIE) step. Microchannels (50 μm wide, 1 μm deep) for interfacing the nanochannels to sand-blasted loading ports were created via a UV contact lithography followed by RIE. The etched substrates were directed bonded to 0.17 μm fused silica coverglass via a direct bonding process based on RCA cleans.

The nanochannel depth is 97 nm, as measured by atomic force microscopy (AFM) in the 700 nm and 1000 nm wide region of a duplicate device from the same wafer. The widths of the nanochannels in the cascade were measured by scanning electron microscopy (SEM) in this duplicate device. The SEM images and cross-section measurements are included in the supplementary material.³²

B. DNA preparation

λ -DNA (48.5 kilobase pairs, kbp, New England Biolabs) was stained with YOYO-1 fluorescent dye (Invitrogen) at a concentration of 1 dye molecule for every 10 bp of DNA. After staining at room temperature for an hour, the DNA samples were heated to 50 °C for 12 h to make the distribution of the dye molecules more homogeneous.^{33,34} DNA (10 $\mu\text{g}/\text{ml}$) samples were prepared in $1 \times$ (10 mM) Tris-base buffer. Prior to nanochannel experiments, an oxygen scavenger β -mercaptoethanol (BME, Sigma-Aldrich) was added to the solution (6% v/v) to suppress photobleaching.

To calculate the ionic strength of the solution, the system of chemical equilibrium equations was solved iteratively (Tris base: $\text{pK}_b = 5.96$, BME: $\text{pK}_a = 9.6$) using a program developed by Santiago and co-workers,³⁵ leading to an ionic strength $I = 7.18$ mM and $\text{pH} = 7.49$. The available on-line program³⁵ was modified to calculate ionic strength of a buffer according to a method used by Hsieh *et al.*;³¹ we confirmed the accuracy of the modified program by reproducing data previously appearing in the literature.³¹ Experimentally measured pH values were also comparable to the predicted value, further confirming the accuracy of the calculation. We chose to work at this low ionic strength to access relatively large values of the fractional extension of λ -DNA in these channel sizes,^{18,36} which greatly simplifies the image analysis. The lowest value of ionic strength was limited by the use of an anti-photobleaching agent (BME), which increases the ionic strength through protonic dissociation.³¹ We used the same method to compute the ionic strength for data appearing in the literature,^{5,37} using the reported buffer conditions.

We use λ -DNA for convenience, as it is readily available and less susceptible to shear cleavage than longer DNA.³⁸ Recently, we have shown that λ -DNA in a high ionic strength buffer ($l_p = 53$ nm, $w = 4.6$ nm) is long enough so that its mean span X is essentially in the asymptotic limit for long chains in square nanochannels up to $D_{\text{Av}} = 400$ nm.²⁸ This conclusion extends to lower ionic strengths²⁸ because the effective width and persistence length have different

dependencies on ionic strength.²⁷ A detailed justification for this argument is given in the supplementary material.³²

C. Nanochannel experiments

Two parallel microchannels on the device were loaded with DNA solution by capillary action. Each reservoir on the device was then sealed with one of the top ends of a T-junction. The other top end was used for both filling up the reservoir and for introducing fresh DNA solution, which was done every 3 h. The bottom end of the T-junction was used to apply pressure by connecting it to a two-way valve having a pressurized air line on one side and atmospheric pressure on the other side. Four T-junctions were assembled into a chuck.³⁹ After filling up the reservoirs with DNA solution and sealing the other top end of the T-junction with the help of O-rings, DNA molecules were drawn inside the nanochannels by applying pressure on the bottom end of two T-junctions directly connected to the reservoirs adjacent to the 1000 nm wide nanochannel region. Once the DNA reached a desired location inside the nanochannels, the pressure in all the four reservoirs was brought down to atmospheric pressure by using all four valves.

The locations of each 45 μm long section of constant width D_2 were recorded from calibration marks and accessed using a Prior II stage controller, which has a precision of 2 μm . To ensure we are not at the interface, we only use data in the central 40 μm of a region of constant width. To make a measurement in a given channel width D_2 , a fresh group of molecules were brought from the reservoir to the center of that region of the nanochannel. The DNA thermal fluctuations were recorded with an EM-CCD camera (Photometrics, Cascade II) using a $100\times$ (1.4 N.A.) oil immersion objective on an inverted epifluorescence microscope (Leica DMI 4000B) with a 120 W metal halide light source at 75% of maximum intensity. We used Micro-Manager to record 1500 images at a speed of 10 frames per second with 100 ms exposure time. We chose to observe many molecules at a fixed channel size, rather than tracking single DNA molecules as they move through the cascade, to increase the throughput. We also found that tracking single molecules with such long exposure times led to significant photobleaching after 150 s.

D. Data processing

Molecule extensions were extracted from the images using a fitting program developed previously.² The intensity profiles parallel to the channel axis were assumed to follow a double error function profile, a convolution of an error function, and a box function. Using this fit, the molecule extension was estimated at the full width at half max (FWHM) of the fluorescence intensity. This method leads to an uncertainty of roughly 5 pixels to the FWHM, which corresponds to approximately 800 nm. The uncertainty varies from frame to frame and between channel sizes, and we use the latter conservative error estimate for all of our analysis. The mean of the extension for each channel size was calculated by pooling the molecules together under the assumption of homogeneous

contour length. This also added uncertainty since there were undoubtedly some fragmented molecules, due to fractionation within the stock solution, photocleavage, and shear cleavage. Obviously fractionated molecules were removed from the ensemble, but fluctuations in the chain extension make it difficult to determine if some molecules are slightly too short or simply tend to sample the smaller range of extensions during the measurement. There is also some uncertainty from the channel roughness, but we have not included this in our estimate of the error since the fitting uncertainty is the dominant factor. The fluctuations of the extension were calculated by first computing the variance for each individual molecule and then taking the average of these data.

E. Relaxation time

There are two possible approaches to assess the fluctuations of DNA confined in the channel: (i) the variance of the extension and (ii) the longest relaxation time of the chain. We prefer the former approach, since it directly accesses information about the effective spring constant of the confined chain while circumventing subtle details surrounding the hydrodynamics of confined DNA.¹³ Indeed, the hydrodynamics of confined DNA in rectangular channels remains unexplored, and the coupling between the chain configurations and hydrodynamic interactions in rectangular channels should be even more complicated than the case for a square channel. Nevertheless, it is still essential that we obtain an estimate of the relaxation time of the chain to ensure that the data in our ensemble of configurations are uncorrelated. While we could use correlated data and then correct for the correlation error,⁴⁰ the uncorrelated approach is simpler and does not require an accurate measurement of the relaxation time to estimate the additional error in the data analysis due to these correlations.

To compute the relaxation (correlation) time for the extension data, we first computed the autocorrelation function for the instantaneous chain extension fluctuation,

$$G(\tau) = \frac{\langle \delta X(t) \delta X(t + \tau) \rangle_t}{\langle (\delta X(t))^2 \rangle_t} \quad (1)$$

using all of the data for a particular DNA molecule. In the latter, $\delta X = X - \langle X \rangle_t$ where $\langle \cdots \rangle_t$ denotes a time average. The longest relaxation time, τ_R , was obtained by fitting the short-time decay of the autocorrelation function with an exponential function, where the τ_R is the time constant for the exponential. The fit was constrained such that $G(0) = 1$.

Two examples of the autocorrelation are plotted in Fig. 3(a) ($D_2 = 100$ nm) and Fig. 3(b) ($D_2 = 1000$ nm). It is clear that the frame rate used in these experiments, which is dictated by the intensity of the illumination, the field of view of the camera, and the camera sensitivity, is much too slow to make an accurate measurement of the short relaxation time in the 100 nm channel. We chose to use a wide field of view to allow us to image multiple molecules at the same time, which is a compromise between camera frame rate and experimental expediency. As the channel size increases, the data acquisition rate appears to lead to a reasonably good estimate of the relaxation time. The insets of these figures show the autocorrelation time over first 10 s of the fitted data.

FIG. 3. (a) Example of the autocorrelation function $G(\tau)$ versus the time lag τ for one DNA molecule in the 100 nm wide channel. (b) Same data for a DNA molecule in the 1000 nm wide channel.

To ensure that our ensembles of extension measurements consist of uncorrelated data, we used frames spaced by ten times the maximum relaxation time of 1 s. This corresponds roughly to the largest time lags in the insets of Figs. 3(a) and 3(b), which are clearly decorrelated even for the largest channel. With this conservative estimate for the decorrelation time, each DNA molecule contributes 14 extension measurements. The uncorrelated extension data obtained for all the molecules were then pooled to calculate the mean extension for each channel size.

III. SIMULATION METHODS

A. Discrete wormlike chain model

We used an off-lattice, touching-bead, discrete wormlike chain (DWLC) model to perform our PERM simulations.^{27,41} The model consists of a discretized chain of contour length L with $N_b + 1$ beads connected by rigid rods of length l_b . The bond angles between the rods are constrained using the bending potential

$$\beta U_b = \kappa \sum_{j=1}^{N_b-1} (1 - \cos \phi_j), \quad (2)$$

where ϕ_j is the bond angle at the $j + 1$ th bead and κ is the bending constant. Here $\beta = (k_B T)^{-1}$ is the inverse of the thermal energy. The bending constant for a desired persistence

length l_p was obtained by solving the nonlinear equation⁴²

$$\frac{2l_p}{l_b} = \frac{\kappa - 1 + \kappa \coth \kappa}{\kappa + 1 - \kappa \coth \kappa}. \quad (3)$$

Excluded volume interactions in the model are accounted for by the use of a hard-core repulsive potential given by

$$\beta U_{EV} = \begin{cases} \infty & \text{if } r_{ij} \leq w, \\ 0 & \text{if } r_{ij} > w, \end{cases} \quad (4)$$

where r_{ij} is the distance between beads with indices i and j . The minimum distance between the walls and the center of the beads is kept at $w/2$, thereby accounting for the repulsion between the chain and the walls. We used $l_b = w$, as this value of l_b yields sufficient coarse-graining without compromising the physical properties of the model.^{27,28}

B. Parameter estimation

In order to map our simulation results with the DWLC model to experimental results for DNA confined in channels, choosing appropriate parameters for l_p and w at a given ionic strength is of utmost importance. The intercalating dyes in these experiments introduce an additional unknown parameter, the contour length in the presence of the dye. Although many studies have investigated the physical properties of DNA in the presence of these dyes,^{34,43,44} there exists a lack of consensus on how l_p and w vary as a function of dye concentration. Therefore, we chose the simplest approach and estimated the parameters, l_p and w using established theories^{29–31} for undyed DNA. The use of such parameters for l_p and w in the past led to simulation results which agree with experimental data for unconfined, undyed DNA, although there exist systematic errors in predicting properties of dyed DNA.²⁷ This disagreement is conceivably due to the modified contour length, which we use here as a fitting parameter to match our experiments.

The persistence length of DNA is often estimated using either the predictions from Odijk⁴⁵ and Skolnick and Fixman⁴⁶ (OSF) or Dobrynin.³⁰ Although both predictions are in agreement for high ionic strengths ($I > 100$ mM), the persistence length values from the two theories are distinctly different for lower ionic strengths.³¹ The disparity is due to the difference in the predictions for the electrostatic contribution to the persistence length. However, Dobrynin's theory conforms with experimental observations that the electrostatic persistence length $l_p^{\text{el}} \sim k^{-1}$, where k is the Debye length.³¹ Therefore, we made use of the expression for DNA given in Eq. (7) of Ref. 31,

$$l_p = 46.1 + \frac{1.9195}{\sqrt{I}} \text{ nm}, \quad (5)$$

which is based on Dobrynin's theory.³⁰ Equation (5) yields a value of $l_p = 68.7$ nm for the ionic strength corresponding to our experiments.

To model the repulsive electrostatic interactions between distal segments of the chain, Stigter²⁹ obtained an expression for an effective hard core width of DNA by relating the second-virial coefficient of hard cylinders to that arising from

solving the Poisson-Boltzmann equation for DNA. We employed Stigter's theory^{29,31} to obtain the width $w = 18.7$ nm of the DNA molecule at our ionic strength.

C. Pruned-enriched Rosenbluth method

We used the DWLC model with the parameters thus obtained to carry out PERM simulations.^{25–28} We performed chain growth starting with one bead chosen at a random position inside the channel, to a maximum length of $N_b + 1$ beads. Our simulations consisted of 400 000 such tours^{27,28} for each channel size. All averages of metrics such as the extension and its variance were computed on the fly during run-time, which enables growth of long chains, circumventing the clock time required for read-write of large amounts of data. For more details on the implementation of our simulations, such as weighting of configurations and strategies for pruning and enrichment, we direct the reader to previous publications from our group.^{27,28}

In all of the simulations, the axis of the channel passing through its center was set as the x -axis of our coordinate system. Accordingly, the extension of DNA inside nanochannels is the mean span of the chain parallel to the channel axis

$$X \equiv \langle \max(x_j) - \min(x_j) \rangle, \text{ where } (j \in [1, N_b + 1]), \quad (6)$$

where $\langle \dots \rangle$ stands for the weighted average over all the tours.^{27,28} Similarly, we calculated the variance of the extension,

$$\delta^2 X = \langle X^2 \rangle - \langle X \rangle^2 \quad (7)$$

using an online incremental algorithm⁴⁷ to obtain an estimate of the fluctuations of the extension in one pass.

IV. RESULTS

Figure 4 plots both the experimental and simulation data for the average chain extension X as a function of the channel size. The figure illustrates our main points: (i) the experimental data and simulation data are in quantitative agreement to within the experimental uncertainty using a dyed contour length of $18.6 \mu\text{m}$, corresponding to a 13% increase compared to naked DNA; and (ii) the confinement spectroscopy approach introduces only a small systematic error in X as the channel aspect ratio increases up to a relatively large value of $D_2/D_1 = 10$. We have also provided the data in Fig. 4 in tabulated form in the supplementary material³² for clarity.

To compare with previous experiments, we have computed the apparent power law exponents for the rectangular channel data over the full range of channel sizes. From a theory perspective, the use of apparent power laws is fraught with peril, as the values of D_{Av} used here should span from the transition regime, $X \sim D_{\text{Av}}^{-1}$, into the extended de Gennes regime, $X \sim D_{\text{Av}}^{-0.7}$. Indeed, recognizing the existence of two power laws over the relatively narrow range of channel sizes used in DNA experiments was critical to resolving¹² the discrepancy between the early experiments² and blob theory.³ Nevertheless, apparent power law fits are a useful *empirical* approach to compare different experimental data sets. Our data produce a fit $X \sim D_{\text{Av}}^{-0.86 \pm 0.08}$ for the experiments and

FIG. 4. Molecule extension in channels of depth 100 nm and widths 100 to 1000 nm. The mean extension is scaled to a fractional extension using a dyed DNA contour length of $18.6 \mu\text{m}$. Black squares and trendline were calculated using the DWLC model for square channels; blue triangles and trendline were calculated using the DWLC model for rectangular channels; red circles and trendline are experimental data. Experimental data points are the mean of 196 measurements made from 14 molecules. Error bars for experimental data represent the standard error of the mean plus the measurement uncertainty in Sec. II D; mean extension simulation error bars are within the size of the markers. Slopes shown were calculated using a linear regression of the means, with the exponent for the experiments being -0.86 ± 0.08 , the rectangular simulations -0.83 ± 0.02 and the square simulations -0.91 ± 0.03 . Extension is shown on a log-log plot. For clarity, these data are also provided in tabulated form in the supplementary material.³² The inset shows the ratio of the simulated extension in the rectangular channel, X_R , relative to the square channel, X_S , as a function of the aspect ratio, D_2/D_1 .

$X \sim D_{\text{Av}}^{-0.83 \pm 0.02}$ for the simulations. As we might expect, the experimental data are scattered about the trendline with $R^2 = 0.97$, whereas the simulation data are much less noisy ($R^2 = 0.99$). The apparent exponents here are quite similar to results reported previously. For example, the experiments by Reisner *et al.*² in relatively low aspect ratio channels were fit by $X \sim D_{\text{Av}}^{-0.85}$. Previous confinement spectroscopy exper-

iments in tapered nanochannels also produced $X \sim D_{\text{Av}}^{-0.85}$ for λ -DNA,^{4,5} as well as $X \sim D_{\text{Av}}^{-0.83}$ and $X \sim D_{\text{Av}}^{-0.78}$ for T4 DNA (169 kbp).⁴

Figure 5(a) shows the simulated variance in the chain extension, $\delta^2 X$, as a function of the channel size for the square nanochannels. The shape of this curve is essentially the same as obtained previously⁴⁸ in a model of experiments in high ionic strength buffers.² The variance drops precipitously as the channel size decreases below $D_{\text{Av}} \approx 2l_p$. For the larger channels, the insensitivity of $\delta^2 X$ to channel size, $\delta^2 X \sim D_{\text{Av}}^0$, is indicative of the scaling predicted for the extended de Gennes regime¹² and consistent with previous simulations for these channel sizes.^{17,48} Indeed, the observation of a scaling $X \sim D_{\text{Av}}^{-0.7}$ and $\delta^2 X \sim D_{\text{Av}}^0$ over the same range of channel sizes is compelling evidence of an extended de Gennes regime.¹⁷

V. DISCUSSION

A. Regime mixing during confinement spectroscopy

The most interesting part of our analysis is the role of the aspect ratio. It is apparent in Fig. 4 that there is a gradual deviation between the simulation data for the average extension in rectangular nanochannels and the “equivalent” square nanochannels as the aspect ratio increases. However, this deviation is not especially large and somewhat exaggerated by the logarithmic scale used for the power law fits. The inset of Fig. 4 shows how the ratio of the extension in the rectangular channel deviates from its square counterpart as the aspect ratio increases. While there is some error introduced by the aspect ratio, this error is an acceptable compromise with respect to the experimental expediency of confinement spectroscopy. Moreover, the congruence between the extension data we have obtained here, previous data for relatively small aspect ratios,² and previous data for tapered nanochannels^{4,5} indicates that the non-equilibrium

FIG. 5. Molecule extension fluctuations in channels of depth 100 nm and widths 100 to 1000 nm. Mean extension variance has been scaled to a contour length of $L = 18.6 \mu\text{m}$ and a persistence length $l_p = 68.7 \text{ nm}$. (a) Black squares were calculated using the DWLC model for square channels. Error bars are smaller than the symbol size. (b) Blue triangles were calculated using the DWLC model for rectangular channels; red circles are experimental data. Experimental data points are the mean of 14 molecules. Error bars represent the standard error of the mean for rectangle simulations and the standard error of the mean plus the measurement uncertainty in Sec. II D. Both panels are semi-log plots. The dashed line in both panels represents the approximate plateau of the variance for simulated square channels in panel (a).

nature of the tapered nanochannels is probably not a critical factor for measurements of the average extension. However, one should be cautious when interpreting the significance of an apparent scaling exponent extracted from confinement spectroscopy; in the present case, the square channels were best fit by $X \sim D_{\text{Av}}^{-0.91 \pm 0.03}$ ($R^2 = 0.99$), which is substantially different than the exponent -0.83 ± 0.02 obtained for simulations in the rectangular channels.

While the average extension, X , is not strongly affected by the channel aspect ratio, this is not the case for its variance, $\delta^2 X$. Scaling theories can provide insights into this increase in variance in rectangular channels with increasing channel width. Odijk⁶ suggested the square channel size D_{**} for the cross-over between the extended de Gennes and the de Gennes regimes is of the order of l_p^2/w . Assuming a prefactor of unity, this would correspond to a value of $D_{**} \approx 250$ nm for our ionic strength. We also know that the crossover from the transition regime to the extended de Gennes regime should be at $D_* \approx 2l_p = 135$ nm. Recall that all but the two smallest square channels³² used in Fig. 5 should be in the extended de Gennes regime, where $\delta^2 X \sim D_{\text{Av}}^0$.¹⁷ Our data in Fig. 5(a) show excellent agreement with this prediction.

However, recall that for the rectangular channels used in our experiments, the channel width varies between $D_2 = 100$ nm and 1000 nm, while the depth is kept constant at $D_1 = 100$ nm. This would mean that while the depth of the channels severely constrains bending of the molecule, as we would expect in the transition regime, the width of most of the channels used here ($D_2 > 300$ nm) imposes constraints such as those in the de Gennes regime (Fig. 1). In fact, a power law fit to our simulation data for rectangular channels, included in the supplementary material,³² shows that $\delta^2 X \sim D_2^{0.32 \pm 0.12}$ which is strikingly close to the $1/3$ exponent that one would expect in the de Gennes regime.

This mixing of regimes leads to a monotonic increase in the variance for the rectangular channels. In high aspect ratio channels, a fluctuation leading to an increase in the chain extension can be resolved without an increase in excluded volume interactions by swelling of the chain in the direction perpendicular to the channel axis. For these 100 nm deep channels, there is minimal bending penalty when avoiding excluded volume interactions in the wider direction. As a result, the chain can experience large fluctuations in X without incurring a significant excluded volume penalty. In contrast, for an isotropic channel, similar magnitude density fluctuations in the axial direction must lead to substantial excluded volume interactions, which are energetically unfavorable. Moreover, the excluded volume interactions could only be resolved by bending the chain, which is more difficult when both directions are small versus the case where one direction is small and the other is large. The excluded volume interactions, coupled to the bending energy, thereby suppress large fluctuations in X in the smaller aspect ratios.

B. Comparing simulation and experiment

One of the most challenging aspects of simulating DNA thermodynamics in nanochannels is determining the persistence length, effective width, and contour length of dyed

DNA. In our analysis, we treat the contour length of the DNA backbone as the sole free parameter, with the persistence length³⁰ and effective width²⁹ obtained theoretically using the buffer ionic strength. This one degree of freedom allows us to shift the ordinate value of all of the simulation data by a fixed amount, which requires that the overall trend in the simulation data match that of the experiments. Previous studies of DNA extension under dynamic conditions, such as in flow⁴⁹ or electrokinetic stretching around solid obstacles,^{50,51} suggest that the backbone length should increase by 25% to 30% at full dye loading, corresponding to an increase from the naked contour length of 16.5 μm , based on a rise of 0.34 nm per base pair, to a stained contour length around 22 μm .³⁷ At the dye to base pair ratio used here of 1:10, others^{4,43} suggested that the increased contour length due to dye intercalation is approximately 19.8 μm . Alternatively, if we assume that the increase in the backbone length is linear in dye loading up to the maximum loading, then the 40% loading we used here should lead to a dyed contour length of $L = 18.3 \mu\text{m}$.²

Arguably, the best approach to determine the increased extension due to intercalation is to work with concatemers of λ -DNA, on the assumption that the dye increases the contour length uniformly along the chain. While we do not have such data for our experiments, Tegenfeldt *et al.*³⁷ previously reported data for the extension of λ -DNA concatemers $n = 1, 2, \dots, 8$ in 100 nm $\times$ 200 nm channels. In the latter reference, the authors suggested that their dye loading should be close to full intercalation, and assumed a contour length of 22 μm per concatamer. By using PERM simulation results, we found excellent agreement between the simulations and these experiments,³⁷ using a dyed contour length of $L = 20.1 \mu\text{m}$ per λ -DNA concatemer. The alternate choice of $L = 22 \mu\text{m}$ leads to systematic deviations between the simulations and experiments. A detailed comparison is included in the supplementary material.³²

Nyberg *et al.*³⁴ provide two possible explanations for this smaller than expected extension due to intercalation. First, their findings suggest that the amount of DNA extension as a function of YOYO loading depends on the ionic strength of the buffer solution. Intriguingly, DNA extension at lower ionic strengths shows a stronger than linear dependence with YOYO concentration, while the dependence is weaker than linear at higher ionic strengths. This might explain why the modified contour length in the data from Tegenfeldt *et al.*³⁷ is lower than the expected value of $L = 22 \mu\text{m}$, as the ionic strength in their work is high. Second, Nyberg *et al.*³⁴ have shown that the heating protocol that makes the dye ratios homogeneous is more effective at low dye concentrations than at high dye concentrations. Considering that Tegenfeldt *et al.*³⁷ worked with dye ratios close to maximum intercalation, we could expect more heterogeneity leading to an average contour length smaller than the maximum value at complete intercalation.

Given these competing arguments, we chose to run a series of simulations for contour lengths around these estimates and found that a value of $L = 18.6 \mu\text{m}$ most closely agrees with our experimental data in Fig. 4. The difference between our result and other estimates^{2,4,43} is small if we consider

the error in measuring the chain extension from the double-error function fit, especially at the smaller values of the extension.

Overall, the quantitative agreement between the simulations and experiments is excellent considering that there is a single degree of freedom, namely, the increased extension due to intercalation. We use this single fitting parameter to shift the ordinate value for the extension data. Thus, the experimental data and simulation results for the average extension need to have the same functional form to produce quantitative agreement. While the apparent exponents are slightly different, the overall shapes of the curves in Fig. 4 are similar enough to provide satisfactory agreement. For the variance data, the two data sets are clearly in qualitative agreement in Fig. 5(b), and the quantitative agreement is very satisfying when we consider that there are no adjustable parameters remaining to fit the data; we have used the one degree of freedom to set the value of L in Fig. 4. Note that the simulations and experiments only agree for rectangular channels; the data for the variance in square channels in Fig. 5(a) is qualitatively different than for rectangular channels. Finally, we should point out that the quantitative agreement between simulation and experiment is aided by using the actual values of the experimental channel width D_2 rather than the target widths.³²

VI. CONCLUSIONS

In the present contribution, we have used an equilibrium confinement spectroscopy approach to assess the role of the aspect ratio on the mean extension and variance of DNA confined to a nanochannel. The average extension is only weakly affected by the aspect ratio, even out to aspect ratios of ten, making confinement spectroscopy a powerful tool for probing DNA extension by confinement. Moreover, the non-equilibrium nature of a tapered nanochannel is a small perturbation for measurement of the average extension.

Interestingly, our analysis also showed that confinement spectroscopy leads to qualitative differences in the fluctuations of the chain extension as a function of aspect ratio. Indeed, for our highest aspect ratio channels, the “equivalent” square channel should be in the extended de Gennes regime and have scaling $\delta X^2 \sim D_{\text{Av}}^0$.¹⁷ Both our experiments and simulations clearly indicate that the variance in the rectangular nanochannels continues to increase with D_{Av} . As a result, confinement spectroscopy is probably not the best tool for probing the different theories for channel-confined DNA; such experiments would require crossing between different regimes of confinement in the same device, and thus experience the “regime mixing” effects we observed here. These theories could be modified to account for aspect ratio⁶ and then compared to equilibrium confinement spectroscopy data. However, this does not seem to be an optimal approach since it adds another degree of freedom (aspect ratio) onto an already contentious area of research. It seems more promising to pursue ionic strength as the independent thermodynamic variable,^{18,36,52} rather than channel size, with a modest number of approximately square channel of different width to provide overlapping data. It is also possible to create a single

device with many square channels, for example, by using a focused ion beam.⁵³

These findings have important consequences for use of nanochannels for high-throughput DNA mapping. All other considerations being equal, when comparing devices that yield the same DNA extension, the device with the lower fluctuations is to be preferred as fluctuations may adversely impact barcode alignment. This is one factor that might make aspect ratio unity nanochannels preferable to slit-like nanochannels. Naturally, there are a number of other factors involved in choice of nanochannel design, including one’s available fabrication resources/process cost, confinement free energy, and hydrodynamic impedance.

While our focus here is on the role of the aspect ratio on the thermodynamic properties of confined DNA, we should also point out the very good quantitative agreement between experiment and simulation for the rectangular nanochannels. The only inputs to the model are the ionic strength of the buffer, which sets the chain persistence length and effective width via established theories,^{29,30} and the dyed contour length of the chain, which we determined from a best fit to the experimental data and obtained a result that is well within the range of other independent experiments. Importantly, there are no adjustable parameters to fit our experimental data for the variance. The results thus obtained lend confidence to simulation predictions and theories of confined DNA based on the wormlike chain model. We are thus optimistic that experiments that carefully control for the role of the channel aspect ratio will be able to resolve many of the outstanding questions surrounding the theory of channel confined DNA.

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¹W. Reisner, J. N. Pedersen, and R. H. Austin, *Rep. Prog. Phys.* **75**, 106601 (2012).

²W. Reisner, K. J. Morton, R. Riehn, Y. M. Wang, Z. Yu, M. Rosen, J. C. Sturm, S. Y. Chou, E. Frey, and R. H. Austin, *Phys. Rev. Lett.* **94**, 196101 (2005).

³M. Daoud and P.-G. de Gennes, *J. Phys. (Paris)* **38**, 85 (1977).

⁴F. Persson, P. Utiko, W. Reisner, N. B. Larsen, and A. Kristensen, *Nano Lett.* **9**, 1382 (2009).

⁵E. Werner, F. Persson, F. Westerlund, J. O. Tegenfeldt, and B. Mehlig, *Phys. Rev. E* **86**, 041802 (2012).

- ⁶T. Odijk, *Phys. Rev. E* **77**, 060901(R) (2008).
- ⁷T. Odijk, *Macromolecules* **16**, 1340 (1983).
- ⁸Y. Yang, T. W. Burkhardt, and G. Gompper, *Phys. Rev. E* **76**, 011804 (2007).
- ⁹T. W. Burkhardt, Y. Yang, and G. Gompper, *Phys. Rev. E* **82**, 041801 (2010).
- ¹⁰P. Cifra, *J. Chem. Phys.* **131**, 224903 (2009).
- ¹¹P. Cifra, Z. Benková, and T. Bleha, *J. Phys. Chem. B* **113**, 1843 (2009).
- ¹²Y. Wang, D. R. Tree, and K. D. Dorfman, *Macromolecules* **44**, 6594 (2011).
- ¹³D. R. Tree, Y. Wang, and K. D. Dorfman, *Phys. Rev. Lett.* **108**, 228105 (2012).
- ¹⁴D. R. Tree, Y. Wang, and K. D. Dorfman, *Phys. Rev. Lett.* **110**, 208103 (2013).
- ¹⁵L. Dai, J. van der Maarel, and P. S. Doyle, *Macromolecules* **47**, 2445 (2014).
- ¹⁶F. Brochard-Wyart, T. Tanaka, N. Borghi, and P.-G. de Gennes, *Langmuir* **21**, 4144 (2005).
- ¹⁷L. Dai and P. S. Doyle, *Macromolecules* **46**, 6336 (2013).
- ¹⁸K. Jo, D. M. Dhirga, T. Odijk, J. J. de Pablo, M. D. Graham, R. Runnheim, D. Forrest, and D. C. Schwartz, *Proc. Natl. Acad. Sci. U.S.A.* **104**, 2673 (2007).
- ¹⁹S. K. Das, M. D. Austin, M. C. Akana, P. Deshpande, H. Cao, and M. Xiao, *Nucleic Acids Res.* **38**, e177 (2010).
- ²⁰R. L. Welch, R. Sladek, K. Dewar, and W. W. Reisner, *Lab Chip* **12**, 3314 (2012).
- ²¹E. T. Lam, A. Hastie, C. Lin, D. Ehrlich, S. K. Das, M. D. Austin, P. Deshpande, H. Cao, N. Nagarajan, M. Xiao, and P.-Y. Kwok, *Nat. Biotechnol.* **30**, 771 (2012).
- ²²A. R. Hastie, L. Dong, A. Smith, J. Finklestein, E. T. Lam, N. Huo, H. Cao, P.-Y. Kwok, K. R. Deal, J. Dvorak, M.-C. Luo, Y. Gu, and M. Xiao, *PLoS One* **8**, e55864 (2013).
- ²³K. D. Dorfman, S. B. King, D. W. Olson, J. D. P. Thomas, and D. R. Tree, *Chem. Rev.* **113**, 2584 (2013).
- ²⁴R. Marie, J. N. Pederson, D. L. V. Bauer, K. H. Rasmussen, M. Yusuf, E. Volpi, H. Flyvbjerg, A. Kristensen, and K. U. Mir, *Proc. Natl. Acad. Sci. U.S.A.* **110**, 4893 (2013).
- ²⁵P. Grassberger, *Phys. Rev. E* **56**, 3682 (1997).
- ²⁶T. Prellberg and J. Krawczyk, *Phys. Rev. Lett.* **92**, 120602 (2004).
- ²⁷D. R. Tree, A. Muralidhar, P. S. Doyle, and K. D. Dorfman, *Macromolecules* **46**, 8369 (2013).
- ²⁸A. Muralidhar, D. R. Tree, Y. Wang, and K. D. Dorfman, *J. Chem. Phys.* **140**, 084905 (2014).
- ²⁹D. Stigter, *Biopolymers* **16**, 1435 (1977).
- ³⁰A. V. Dobrynin, *Macromolecules* **39**, 9519 (2006).
- ³¹C.-C. Hsieh, A. Balducci, and P. S. Doyle, *Nano Lett.* **8**, 1683 (2008).
- ³²See supplementary material at <http://dx.doi.org/10.1063/1.4879515> for SEM images of the channel cross section, details on the role of molecular weight, tabulated data for Fig. 4, the power law fit for the variance in the mean extension in rectangular channels, and more details on the increased extension due to intercalation.
- ³³C. Carlsson, M. Jonsson, and B. Åkerman, *Nucleic Acids Res.* **23**, 2413 (1995).
- ³⁴L. Nyberg, F. Persson, B. Åkerman, and F. Westerlund, *Nucleic Acids Res.* **41**, e184 (2013).
- ³⁵See http://microfluidics.stanford.edu/download/CalcEquilibrium_LoC_v1p1.m for the program for equilibrium reaction calculations.
- ³⁶W. Reisner, J. P. Beech, N. B. Larsen, H. Flyvbjerg, A. Kristensen, and J. O. Tegenfeldt, *Phys. Rev. Lett.* **99**, 058302 (2007).
- ³⁷J. O. Tegenfeldt, C. Prinz, H. Cao, S. Chou, W. W. Reisner, R. Riehn, Y. M. Wang, E. C. Cox, J. C. Sturm, P. Silberzan, and R. H. Austin, *Proc. Natl. Acad. Sci. U.S.A.* **101**, 10979 (2004).
- ³⁸R. T. Kovacic, L. Comai, and A. J. Bendich, *Nucleic Acids Res.* **23**, 3999 (1995).
- ³⁹F. Persson and J. O. Tegenfeldt, *Chem. Soc. Rev.* **39**, 985 (2010).
- ⁴⁰J. D. Chodera, W. C. Swope, J. W. Pitera, C. Seok, and K. A. Dill, *J. Chem. Theory Comput.* **3**, 26 (2007).
- ⁴¹J. Wang and H. Gao, *J. Chem. Phys.* **123**, 084906 (2005).
- ⁴²J. Schellman, *Biopolymers* **13**, 217 (1974).
- ⁴³K. Günther, M. Mertig, and R. Seidel, *Nucleic Acids Res.* **38**, 6526 (2010).
- ⁴⁴M. L. Bennink, O. D. Schärer, R. Kanaar, K. Sakata-Sogawa, J. M. Schins, J. S. Kanger, B. G. de Grooth, and J. Greve, *Cytometry* **36**, 200 (1999).
- ⁴⁵T. Odijk, *J. Polym. Sci.: Polym. Phys. Ed.* **15**, 477 (1977).
- ⁴⁶J. Skolnick and M. Fixman, *Macromolecules* **10**, 944 (1977).
- ⁴⁷D. H. D. West, *Commun. ACM* **22**, 532 (1979).
- ⁴⁸D. R. Tree, Y. Wang, and K. D. Dorfman, *Biomicrofluidics* **7**, 054118 (2013).
- ⁴⁹T. T. Perkins, D. E. Smith, R. G. Larson, and S. Chu, *Science* **268**, 83 (1995).
- ⁵⁰O. B. Bakajin, T. A. J. Duke, C. F. Chou, S. S. Chan, R. H. Austin, and E. C. Cox, *Phys. Rev. Lett.* **80**, 2737 (1998).
- ⁵¹G. C. Randall and P. S. Doyle, *Macromolecules* **38**, 2410 (2005).
- ⁵²C. Zhang, F. Zhang, J. A. van Kan, and J. R. C. van der Maarel, *J. Chem. Phys.* **128**, 225109 (2008).
- ⁵³L. D. Menard, and J. M. Ramsey, in *Proceedings of MicroTAS 2013*, edited by R. Zengerle, A. Manz, P. Schuille, and H. Becker (Transducer Research Foundation, Freiburg, Germany, 2013).