We demonstrate a new platform, convex lens-induced nanoscale templating (CLINT), for dynamic manipulation and trapping of single DNA molecules. In the CLINT technique, the curved surface of a convex lens is used to deform a flexible coverslip above a substrate containing embedded nanotopography, creating a nanoscale gap that can be adjusted during an experiment to confine molecules within the embedded nanostructures. Critically, CLINT has the capability of transforming a macroscale flow cell into a nanofluidic device without the need for permanent direct bonding, thus simplifying sample loading, providing greater accessibility of the surface for functionalization, and enabling dynamic manipulation of confinement during device operation. Moreover, as DNA molecules present in the gap are driven into the embedded topography from above, CLINT eliminates the need for the high pressures or electric fields required to load DNA into direct-bonded nanofluidic devices. To demonstrate the versatility of CLINT, we confine DNA to nanogroove and nanopit structures, demonstrating DNA nanochannel-based stretching, denaturation mapping, and partitioning/trapping of single molecules in multiple embedded cavities. In particular, using ionic strengths that are in line with typical partitioning/trapping of single molecules in multiple embedded cavities, we have successfully extended DNA in sub-30-nm nanochannels, achieving high stretching (90%) that is in good agreement with Odijk deflection theory, and we have mapped genomic features using denaturation analysis.

convex lens-induced nanoscale templating | polymer confinement | genomic mapping | CLIC imaging | nanotechnology

Nanoconfinement-based manipulation is a powerful approach for controlling the conformation of single DNA molecules on chip. When single polymer chains are squeezed into environments confined at length scales below their diameter of gyration in free solution, the polymer equilibrium conformation will be molded by the surrounding nanoscale geometry. Nanochannel arrays can be used for massively parallel extension of DNA across an optical field, serving as the basis for a high-throughput optical mapping of genomes (1, 2). More varied manipulations can be performed based on the design of the surrounding nanotopology, such as using nanocavities embedded in a nanoslit to trap single DNA molecules (3). Nanoconfinement-based manipulation, compared with competing techniques for single-molecule manipulation such as tweezers technology and surface/hydrodynamic-based stretching, has three key advantages (4): (i) It is highly parallel, providing the high throughput essential for mapping genome-scale mammalian genomes (1); (ii) it can be efficiently integrated with microfluidics to rapidly cycle molecules through the channel arrays for upstream/downstream pre- and postprocessing of DNA; and (iii) it does not require applied flow or electric force to maintain the DNA extension.

Nanoconfinement-based approaches have, however, a key difficulty inherent to the use of nanoscale dimensions: the need to bridge length scales differing by up to 5 orders of magnitude (submillimeter scale of a pipette tip to channels in the 10–100 nm range) in the same fluidic device. This introduces two challenges in device design and manufacture: (i) the need to drive single-molecule analytes across a very high free-energy barrier at the edge of nanofluidic devices and (ii) inefficient fluid transport due to the high hydraulic resistance of channels with nanoscale dimensions.

These challenges have the practical range of nanochannel dimensions to 40–50 nm (1). Moreover, the high hydraulic resistance of nanoscale features (for a slit of depth $h$, the hydraulic resistance scales as $1/h^3$, compared with $1/h$ for electrical resistance) (5) requires that electrophoretic actuation be used to drive DNA into sub-100-nm nanochannels. This in turn necessitates the use of special high-salt electrophoresis buffers [2–5X Tris/borate/EDTA (TBE)], which reduces DNA extension (6) and constrains the imaging buffer used. One answer to these challenges is to develop specialized grayscale lithography approaches (7, 8) that can create gently funneling channel dimensions, reducing the free-energy barrier. Gray-scale approaches, although they are feasible technologically, are still highly challenging to implement and remain limited in the range of confinement that can be varied continuously in both lateral and vertical dimensions.

To overcome the challenges faced by classical nanofluidic technology, we have developed a new approach for introducing tunable nanoscale confinement to trap and align DNA molecules for optical analysis. Our confinement-based imaging technology combines nanotemplated substrates with a single-molecule imaging technique called convex lens-induced confinement (CLIC) (9). Fig. 1 illustrates a flow cell implementation of CLIC microscopy, in which molecules are initially loaded into a planar micron-scale chamber (10). To form the CLIC imaging chamber, the upper chamber surface is subsequently pressed into contact with the lower surface using the curved surface of a lens (Figs. 1B and 2). The final vertical confinement profile varies gradually away from the contact point, typically increasing by tens of nanometers over a field of view of a hundred microns. When CLIC is performed over a surface containing nanotemplated

Significance

Convex lens-induced nanoscale templating (CLINT) represents a conceptual breakthrough in nanofluidic technology for single-molecule manipulation. CLINT solves a key challenge faced by the nanofluidics field by bridging the multiple-length scales required to efficiently bring single-molecule analytes from the pipette tip to the nanofluidic channel. To do this, CLINT loads single-molecule analytes into embedded nanofeatures via dynamic control of applied vertical confinement, which we have demonstrated by loading and extending DNA within nanochannels. CLINT offers unique advantages in single-molecule DNA mapping by facilitating surface passivation, increasing loading efficiency, obviating the need for applied pressure or electric fields, and enhancing compatibility with physiological buffers and long DNA molecules extracted from complex genomes.
structures such as nanocavities and nanogrooves, the vertical confinement imposed by CLIC drives the single-molecule analytes into the embedded topology. We call this approach “convex lens-induced nanoscale templating” (CLINT). Note that in CLINT, the molecules are loaded gently by imposing confinement from above, eliminating the need for high pressures or electric fields to introduce single-molecule analytes into the confined region of the device. This ease of loading ensures that the CLINT approach, when used to confine macromolecules in structures with dimensions smaller than their persistence length, is compatible with a much wider range of ionic conditions than classic direct-bonded devices that rely on electrophoresis for loading. Loading molecules from above can also reduce the sensitivity of the technique to fabrication defects that can lead to clogging of direct-bonded channels loaded from the side. We demonstrate that CLINT can efficiently load DNA into nanochannels less than 30 nm in size, imposing subpersistence length confinement at which stretching is high, polymer back-folding is energetically unfeasible, and thermal fluctuations are suppressed (i.e., the Odijk regime). The ability to operate devices in this regime is critical as the suppression of back-bending and thermal fluctuations leads to more efficient alignment of optically mapped DNA fragments to a reference genome (11). Finally, unlike enclosed classical nanofluidic devices, the CLINT device interior can be easily exposed, leading to greater reusability and ease of access. This feature can be used, for example, to load single cells at precise locations on the chip adjacent to nanofluidic features and to apply surface coatings to suppress nonspecific interactions of fluorescent probes and proteins with exposed device surfaces.

In CLINT imaging, DNA molecules are loaded into a chamber formed by two transparent surfaces, such as a fused silica coverslip and substrate, separated by a spacer (typically 5- to 30-µm-thick adhesive tape). Molecules loaded into the chamber are initially unconfined (Fig. 1A). A convex lens mounted on a nanopositioner presses down on the upper coverslip, deforming it (Figs. 1B and 2B). The upper surface bows downward until it comes into contact with the lower planar coverslip at a single point, forming the CLIC chamber geometry (Fig. 2C). The applied vertical confinement causes DNA molecules to favor extended conformations due to self-exclusion interactions in the region surrounding the contact point, where the chamber height is less than the diameter of gyration Dg of DNA in free solution (4). The lower surface of the imaging chamber contains embedded nano-lithography (e.g., nanochannels) subjecting the DNA to additional transverse confinement. When the chamber height is small enough, the molecules’ free energy is minimized when they are maximally extended in the channels (Fig. 1B), which we observe to occur spontaneously. This technique can be applied to other nanotopologies (e.g., nanopits) and other nanomaterials (e.g., actin, microtubules, DNA nanotubes, nanowires).

When DNA is confined to a nanochannel of dimensions less than Dg, it will stretch out along the channel axis due to excluded-volume interactions. If the width D of a square cross-section nanochannel is larger than the 50 nm persistence length P of the DNA, the polymer is described by the de Gennes confinement regime and will coil up into multiple blobs along the nanochannel axis (4). When D < P, coiling within the channel is suppressed and the molecule undergoes periodic deflections along the walls with no back-bending (Odijk regime) (12).
Experimental Methods

We implement CLINT imaging using the microscopy device shown in Fig. 2A (10), mounted on an inverted fluorescence microscope (SI Text, Imaging Conditions). The CLIC imaging chamber (Fig. 2B) is formed between two confining surfaces. The lower surface is a fused silica substrate containing embossed nanotopography. The upper surface is a coverslip with two small holes sand-blasted into the corners for fluid insertion and removal. Double-sided adhesive tape (30- or 10-μm thick; Nichi Denko) separates the upper and lower chamber surfaces, and is laser-cut (PBS Engraving) to create channels for liquid to flow into a main central chamber (SI Text, Substrate Preparation). Computer-controlled syringe pumps insert and retrieve the sample from the imaging chamber, facilitating serial measurements and sample recovery. Chemically inert fluorinated ethylene propylene tubing connects the pump outlets to the holes in the upper coverslip and a seal is formed between them by a thick silicone gasket.

To form the CLIC imaging chamber, a lens mounted on a nanomotisor presses down on the upper coverslip, causing it to deform around its curved surface (Fig. 2B) (SI Text, Experimental Procedure). The chamber height increases very gradually away from the contact point, e.g., by 15 μm in height over a 60-μm distance from the contact point (using 30-μm-thick adhesive tape, as in Fig. 2C). DNA extension experiments were performed using 30-μm-thick adhesive, and denaturation mapping experiments with 10-μm–thick adhesive.

In studying DNA confinement, it is necessary to measure the chamber height profile precisely (SI Text, Chamber Height Characterization and Figs. 51 and 52). The chamber height profile is measured using both interferometry and fluorescence (10). To perform interferometry, we removed the emission filter from the imaging system and directly imaged the excitation field. When the chamber formed, we observed Newton’s rings (Fig. 2D), which result from the interference of reflections of the laser light from the upper and lower confining surfaces. We image this interference profile over a wide field by taking a series of high-resolution images while moving the sample in a horizontal plane relative to the microscope objective. The intensity of the interference pattern for wavelength λ is minimum at chamber heights corresponding to integer multiples of λ/(2n cos δ), where n is the index of refraction of the medium (in this case water, for which n = 1.33) and δ is the angle of incidence of the laser illumination (45°). Next, we measure the fluorescence intensity of an in situ dye solution (Alexa Fluor 647, Fig. 2E), which emits into a spectrally separate channel from the DNA. The dye fluorescence intensity is proportional to the local chamber height. By fitting the dye intensity profile, and using this function to fit the interference pattern subsequently, we determine the chamber height profile using the method described in ref. 10.

The lower surface of the imaging chamber contains nano- and macroscale lithography. Substrates containing arrays of 27 × 27 × 200,000 nm³, 50 × 50 × 200,000 nm³ nanochannels, and 50 × 600 × 600 nm² and 50 × 900 × 900 nm² nanopits were fabricated. These features were patterned in fused silica using electron-beam lithography (VSB UHR EDF; Vistec Lithography) and etched using reactive ion etching (RIE). One-micron-deep microchannels, which can be easily seen in Fig. 2D and E, were defined using contact UV photolithography (EVG620; EVG) and etched using RIE. The microchannels were designed to enable buffer exchanges when the imaging chamber is compressed, but were not used in these experiments. Shown in Fig. 2F and G are SEM images of the 900-nm nanopits and 50-μm nanochannels, respectively. The spacing between nanopits is 4 μm and the spacing between nanochannels is 2 μm. These nanoscale features were aligned with the push-lens using an XY micropositioning stage before an experiment and before the chamber was formed.

The chamber was first wet using a solution of 45 mM Tris-base, 45 mM boric acid and 1 mM EDTA (0.5x TBE), with 3% (vol/vol) β-mercaptoethanol (BME) added as an antiphotobleaching agent. λ-Phage DNA (48.5 kbp; New England Biolabs) was used at a concentration of 50 μg/mL for nano-channel experiments and T4-phage DNA (166 kbp; New England Biolabs) was used at a concentration of 5 μg/mL for nanopit experiments. Experiments were carried out in 0.5x TBE, with 577 pm Alexa Fluor 647 (to measure the chamber height profile) and an antiphotobleaching system consisting of 3% BME, 5 μM protocatechuic acid, and 500 nM protocatechuic dioxygenase. The DNA was stained with YOYO-1 fluorescent dye (Life Technologies) at a ratio of one dye molecule per 10 bp. YOYO-1 increases the DNA contour length at this staining ratio from 16.5 μm to 19.0 ± 0.7 μm for λ-DNA and from 56.4 μm to 65 ± 2 μm for T4 DNA (SI Text, Sample Preparation) (17). Denaturation mapping experiments were performed using λ-DNA at a concentration of 25 μg/mL, 0.25x TBE buffer, 3% BME, and 50% formamide (2).
Results and Discussion

DNA Extension in Nanochannels. We demonstrate CLINT microscopy by dynamically loading λ-DNA into square nanochannels of D = 27 nm (Fig. 3A) and 50 nm (Fig. 3B). As schematically portrayed in Fig. 1, DNA molecules evolve from bundled to extended conformations as the push-lens is lowered into contact with the lower surface of the chamber. The DNA in Fig. 3B is partially confined to two separate channels in the first and second frames because the spacing between channels is only 2 μm. When the push-lens is lowered further, the molecule becomes completely confined to a single nanochannel (Movie S1). Eq. 1 predicts maximum extended lengths for λ-DNA of 16.8 ± 0.6 μm in a 27-nm channel and 15.7 ± 0.6 μm in a 50-nm channel, consistent with the measured extensions. Here we have assumed a persistence length l_p ≈ 51 ± 3 nm (17).

Quantitative Analysis of Extended DNA. We characterize the physical extension of DNA molecules in the CLINT device as a function of applied vertical confinement. This quantitative characterization is essential to understand the properties of DNA for optical genomic analysis and to optimize the design of our experimental setup and assay conditions. Movies of DNA molecules were acquired at several locations in the CLIC imaging chamber at a frame rate of 11 Hz, for a total of 100 frames. The intensity profile of a DNA molecule extended in a nanochannel is expected to be a step function convolved with a Gaussian point-spread function. This convolution yields a model for the intensity profile I(x, t) of a linearly extended polymer along the channel that is described by the difference of two error functions (4). To extract the extension of individual molecules stretched in the channels, the error function model for the intensity profile was fit to each frame, using a least-squares fitting algorithm. See SI Text, DNA Length Determination and Fig. S3 for greater detail.

We visualize DNA extensions and fluctuations in the channels as a function of chamber height (see Fig. 4B for representative molecules in 27-nm channels at a series of heights). The per-molecule time variation of DNA length is shown in the histograms of Fig. 4B: These are influenced by the chamber temperature and could be minimized by cooling the sample. Fig. 4C plots DNA fractional extension as a function of confinement. Molecules which were obviously photonicicked or stuck to the confining surfaces were not included in the analysis. The DNA are, on average, slightly more extended in the 27-nm channels than in the 50-nm channels at any given chamber height, with the longest molecules located at chamber heights less than 50 nm and having time-averaged lengths of 17.18 ± 0.05 μm and 17.37 ± 0.03 μm, respectively. The majority of the DNA fragments were trapped at a chamber height less than ~100 nm. Fig. 4C compares our measurements directly to Odijk theory and extended Odijk theory which includes S and C loops (16). Note that the maximal extensions obtained at the lowest chamber height compare favorably with Odijk predictions. The deviation away from the deflection theory for higher CLINT heights is consistent with the loop-theory model. Due to the applied confinement gradient and flow field in lowering the lens, all DNA molecules sample a range of chamber heights (SI Text, Variation in Confinement Along Molecules and Figs. S4 and S5). Each molecule’s length fluctuates dynamically with time.

DNA Confinement in Nanopits. The CLINT technique can also be used to confine DNA to other types of nanostructures. We have applied CLINT microscopy to visualize DNA loading and confinement in arrays of 600- and 900-nm square nanopits, etched to a depth of 50 nm. The pits are arranged in a square lattice with a spacing of 4 μm. As in the nanochannel experiments, DNA (in this case T4 DNA, 166 kbp) was injected in the imaging chamber with the push-lens raised. When the push-lens was placed over the nanopit array...
and lowered into contact with the bottom substrate, DNA molecules were driven into the nanopits from above. Due to excluded-volume interactions, DNA molecules adopt conformations with coiled segments stored in multiple pits. The number of pits occupied by each molecule varies with the geometry of the nanopits and the imposed vertical confinement.

Fig. 5A shows T4 DNA being loaded into 600- and 900-nm nanopits. Initially, the molecules are unconfined; as the confinement is dynamically imposed by lowering the push-lens, the DNA molecules occupy an increasing number of nanopits. Molecules confined to 600-nm pits compared with 900-nm pits occupy a higher number of pits because the 900-nm pits can store more of a polymer’s contour (3). Representative images of DNA confined to pits at different chamber heights, at equilibrium, are shown in Fig. 5B. At smaller chamber heights, each DNA molecule extends over a larger number of pits.

**DNA Denaturation Mapping.** To demonstrate genomic mapping using CLINT we performed denaturation mapping of λ-DNA confined to 50 nm channels. AT-rich regions in the DNA melt at a lower temperature than GC-rich regions. The YOYO-1 dye unbinds from the melted regions making it possible to obtain a genomic barcode pattern by imaging the DNA (2). The DNA must be heated to a temperature at which the AT-rich regions melt but the GC-rich regions do not. To perform denaturation mapping, the temperature of the flow cell was raised to 42 °C using a heater located above the push-lens, which corresponded to an approximate sample temperature of 38 °C (SI Text, Denaturation Mapping and Fig. S6). The push-lens was then lowered to confine DNA to the nanochannels. After ~10 s, a barcode-melting pattern consisting of a single dark spot near the center of the molecule became clearly visible. After \( t \approx 40 \text{s} \), a second dark spot appeared as more YOYO-1 was released from a second denatured area (Fig. 6A). Fig. 6B and C shows a partially melted molecule that has reached a steady state ~1 min after lowering the push-lens. Fig. 6D shows the theoretical denaturation barcode for comparison, assuming a helicity of 0.7 (2), confirming good agreement with the experimental data for a single DNA molecule.

**Conclusion**

Classical nanofluidic technology faces several simultaneous challenges: (i) efficient introduction of analytes from macroscale to nanoscale dimensions, (ii) ensuring appropriate surface passivation in nanoscale environments dominated by surface interactions, and (iii) extreme sensitivity to fabrication defects in sub-50-nm channels. The CLINT approach, demonstrated here, addresses these challenges by introducing dynamically controllable confinement. This allows fluidics devices to be transformed in situ from initially macroscale flow cells that enable easy introduction of analytes and passivation chemistries into nanoscale imaging devices, enabling direct single-molecule manipulation and analysis. We demonstrate that DNA can be dynamically loaded from the top and fully extended in sub-30-nm-size channels, as opposed to classic nanochannel approaches which require loading from the side. Top loading, versus side loading, enables introduction of DNA into nanoconfined dimensions with (i) greater efficiency, (ii) control over the rate of analyte introduction by altering the rate of descent of the push-lens (9), and (iii) reduced sensitivity to fabrication defects: One defect can render a side-
loaded channel inoperable, whereas top loading can still use the portion of the channel away from the defect. Reducing sensitivity to defects is critical to enable the cost-effective scaling of nanofluidic technology for easy dissemination and extension to 10-nm dimensions. Previous nanofluidic devices have been based on the need to directly bond nanostructures via high-temperature steps, a need that CLINT obviates. Direct bonding is static and complicates surface passivation approaches critical for working with single-molecule analytes (which tend to stick nonspecifically to surfaces). Moreover, in direct-bonded nanofluidic devices, molecules are introduced into nanochannels from adjoining microchannel reservoirs, requiring high electric or hydrodynamic forces to overcome the large free-energy barriers introduced at the abrupt change in device dimensions.

One powerful advantage of CLINT, demonstrated by capturing of DNA in nanocavity structures, is the ability to dynamically trap single analytes in arrays of local nanowells that are closed off from the surrounding chemical environment, for extended imaging and in the presence of high-reactant concentrations. For example, capturing bound molecular complexes from bulk solution into arrays of nanowells enables their stoichiometry and distribution of properties to be determined. Previous nanoreactor approaches using deformable PDMS lids are fundamentally limited in that the cavities exist in either open or closed states and cannot access a continuous range of confinement conditions (18). For example, CLINT can couple molecularly thin chambers to embedded structures, enabling reagent exchange so that continuous reactions can be performed within the well (e.g., local sequencing). Moreover, CLINT operates with an all-glass structure, so that the fluorescence backgrounds are reduced, and the surfaces are both nanometer-scale smooth and nonporous to oxygen, which facilitates stable imaging. Lastly, CLINT enables working with a much wider range of buffer conditions not limited by the need to prevent surface interactions and create strong electrokinetic driving forces. Thus, buffer conditions can be tuned to optimize imaging and binding conditions or match exact physiological conditions. These advantages suggest that CLINT-based nanofluidics would be an ideal platform for high-throughput mapping of DNA–protein interactions on extended genomes.

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Supporting Information

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SI Text

Sample Preparation
The chamber was first wetted using a solution of 45 mM Tris base, 45 mM boric acid, and 1 mM EDTA (TBE) (0.5x), with 3% β-mercaptoethanol (BME) added as an antiphotobleaching agent. λ-Phage DNA (48.5 kbp; New England Biosciences) was used at a concentration of 50 μg/mL for nanochannel experiments and T4-phage DNA (166 kbp; New England Biosciences) was used at a concentration of 5 μg/mL for nanotip experiments. Experiments were carried out in 0.5× TBE, with 577 pM Alexa Fluor 647 (AF647, to measure the chamber height profile) and an antiphotobleaching system consisting of 5% BME, 5 mM protocatechuic acid, and 500 nM protocatechuate dioxygenase. DNA were stained with YOYO-1 fluorescent dye (Life Technologies) at a staining ratio of one dye molecule per 10 base pairs. YOYO-1 increases the DNA contour length at this staining ratio from 16.5 μm to 19.0 ± 0.7 μm for λ-DNA and from 56.4 μm to 65 ± 2 μm for T4 DNA (1).

Denaturation mapping experiments were performed using λ-DNA at a concentration of 25 μg/mL, 0.25× TBE buffer, 3% BME, and 50% formamide (which lowers the melting temperature) (2).

Substrate Preparation
The fused silica substrates and sand-blasted coverslips were cleaned by sonication in acetone, ethanol, and deionized water. They were then treated with piranha solution (2:1 sulfuric acid to 30% hydrogen peroxide solution) for 30 min and finally with 1 M potassium hydroxide for 15 min.

The imaging flow cell was formed by adhering a fused silica substrate (500-μm thick in Figs. 2–5 and 175-μm thick in Fig. 6) with etched nanochannels to a standard no. 1 coverslip (150-μm thick) using double-sided adhesive [30-μm thick in Figs. 2–5 (no. 5603; Nitto Denko) and 100-μm thick in Fig. 6 (no. 5601; Nitto Denko)]. To create a flow channel and central circular imaging chamber, the adhesive was laser cut using the template delineated by figure 1b in ref. 3.

Imaging Conditions
In Figs. 2–5, imaging was performed using a 60x N.A. 1.49 Nikon oil immersion objective (CFI Plan Apo VC 60XWI) mounted on a Nikon Ti-E microscope. In Fig. 6, a 100x N.A. 1.49 Nikon oil immersion objective (CFI Apo TIRF 100×) was used. An Andor iXon3 EMCCD camera was used with the sensor cooled to −70 °C. DNA images were acquired using 3 mW power from a 488 nm Coherent Sapphire laser, a 50-ms exposure time and an EM gain of 300. In characterizing the chamber geometry, interferometry images were acquired using the same 488-nm laser and ~0.1 mW power; dye images were acquired using a 64-nm Coherent OBIS laser and ~0.1 mW power.

Experimental Procedure
The prepared flow cell was mounted in the convex lens-induced confinement (CLIC) device and passivation buffer was flowed in to wet the chamber. The pusher height required to make coverslip-chip contact was then identified using interferometry. Subsequently, the solution containing the DNA molecules was flowed into the imaging chamber and the pusher-lens was lowered into contact. After waiting ~10 s, which is much longer than the typical conformational stabilization time of DNA in channels of this size (4), movies of the trapped DNA were acquired at a series of locations. After each DNA-imaging experiment, two raster scans (Fig. S1) were performed to characterize the chamber height profile. These scans include direct interferometry and fluorescence imaging of an in situ fluorophore, extending the chamber characterization protocol presented in ref. 3.

Chamber Height Characterization
To precisely quantify DNA confinement, the chamber height was characterized in two steps: by imaging a small in situ fluorophore and by direct interferometry (3). First, the chamber was filled with Alexa Fluor 647 and imaged by scanning the microscope stage to acquire a series of high-resolution images (110-μm wide) in a grid (typically 9 × 9) as displayed in Fig. S1A. A fourth-order, 2D polynomial was then fit to the fluorescence intensity which is proportional to chamber height. Coverslip–coverslip contact was assumed, limited by less than 2 nm surface roughness.

Second, direct chamber interferometry was performed. Specifically, the emission filter was removed to allow observation of the Newton’s rings (Fig. S1B). The chamber height h_m at the nth interference minimum was calculated using Eq. S1 and values for the excitation wavelength (λ = 488 nm), refractive index (n = 1.33), and angle at which the excitation beam leaves the objective (θ = 45° ± 2°). Using this information, the scale factor relating the chamber height to the dye intensity fit was determined. The rescaled dye intensity fit, shown in Fig. S1, provided a measure of the chamber height,

\[
\text{h}_m = \frac{m\lambda}{2n\cos(\theta)}
\]  

For example, cross-sectional fits corresponding to the horizontal and vertical cutlines delineated in Fig. S1B differ in radii of curvature by 4.9%. Repeatedly raising and lowering the lens to reform the chamber yielded a reproducible chamber height profile, as shown by Fig. S2B. The average height profile h(x) along the x axis is characterized by an average radius of curvature of R_{avg} = 129 ± 6 mm, where the stated uncertainty is the SD of R_c.

\[
I(x,y,t) = \frac{I_0}{2} \left[ \text{erf} \left( \frac{y-y_0(t)+\frac{L(t)}{2}}{\sigma \sqrt{2}} \right) - \text{erf} \left( \frac{y-y_0(t)-\frac{L(t)}{2}}{\sigma \sqrt{2}} \right) \right] \times \exp \left[ -\frac{(x-x_0(t))^2}{2\sigma^2} \right] + I_b
\]  

DNA Length Determination
Movies of extended DNA molecules were acquired at a series of locations in the chamber using a frame rate of 11 Hz and a total of 100 frames. A least-squares fit for each frame was calculated in Matlab (MathWorks) to extract the lengths of the extended DNA molecules (Fig. S3). This fit function, which describes the integrated intensity of a DNA molecule along a nanochannel, corresponds to a convolution of a step function (the idealized DNA intensity distribution) with a Gaussian point-spread function (due to diffraction-limited imaging). The fit function is given by Eq. S2, where y is the displacement along the axis of the nanochannel, x is the displacement transverse to the nanochannel axis, t is time, I_0(t) is the peak intensity of the DNA, y_0(t) is the displacement of the center of the DNA along the nanochannel axis, x_0(t) is the displacement of the center of the DNA transverse to the nanochannel axis, L(t) is the length of the
extended DNA molecule, $\sigma$ is the SD of the Gaussian (due to the diffraction limit), $I_0$ is the background intensity, and erf is the error function defined as

$$erf(y) = \frac{2}{\sqrt{\pi}} \int_0^y \exp(-t^2)dt.$$  

**Variation in Confinement Along Molecules**

The DNA extension analysis presented in Fig. 4 used the chamber height measured at the center of each DNA molecule. Here, we consider the effect of the local confinement gradient upon the measured DNA extension. We check to ensure that the change in height along the length of each DNA molecule due to the local confinement gradient results in negligible local modulation of the DNA extension. To do so, we measure the chamber height variation experienced by individual molecules in Fig. 4 and estimate the consequent DNA extension along its length according to theory.

Fig. S4A schematically shows an extended DNA molecule confined in a nanochannel embedded in a CLIC chamber of local height $h$. The chamber height varies continuously along the length of the molecule, differing by a total of $\Delta h$ from end to end. For every DNA molecule presented in Fig. 4 that lies in the region of validity of Odijk theory (i.e., points at $h < 70 \text{ nm}$), the end-to-end $\Delta h$ is measured and plotted in Fig. S4B as a function of the displacement $y$ along the nanochannel axis, measured relative to the contact point. We show that the typical $\Delta h$ over the extended DNA molecules is much less than both the persistence length ($50 \text{ nm}$) and local chamber height; thus we expect the local chamber gradient to have negligible effect upon the DNA extension. The magnitude of the corresponding confinement gradient, $\Delta h/L$, experienced by each molecule along the nanochannel axis, is typically less than $0.5 \times 10^{-3}$ (Fig. S4C).

Fig. S5A delineates the theoretically expected fractional extension $\epsilon$ of a DNA molecule as a function of the chamber height, using the Odijk theory modified to include S and C loops (5), as described in *Confinement Theory*. The total imposed confinement is the sum of the predicted chamber height and the 27-nm depth of the embedded nanochannel. Using the value of $h$ and $\Delta h$ calculated for each data point (in Fig. S5 and Fig. 4), the corresponding variation in extension across each molecule is taken to be $\Delta \epsilon = \epsilon(h - \Delta h/2) - \epsilon(h + \Delta h/2)$. Fig. S5B delineates $\Delta \epsilon$ as a function of chamber height, which is less than one percent in regions where the total imposed confinement is less than 50 nm (chamber height less than 20 nm). In regions where the total imposed confinement is less than 100 nm (chamber height less than 70 nm), the expected variation in extension due to the gradient is still less than 6%, which will have negligible effect on the study of extension as a function of height presented in Fig. 4. In addition, we observed that the fitted width of the extended DNA remained consistent with the diffraction limit and did not vary with chamber height, supporting that the molecules did not spill out laterally from the channels when confined.

The confinement gradient can be further suppressed by reducing the thickness of the adhesive spacer. For example, 30-µm-thick adhesive tape (no. 5603; Nitto Denko) was used to form the flow chambers used for the experiments presented in Fig. 4 and Figs. S4 and S5. Recently, 10- and 5-µm-thick adhesive tape has become available from the same company, allowing the confinement gradient to be reduced by up to a factor of 6. For example, 10-µm-thick adhesive tape (no. 5601; Nitto Denko) was used to collect the denaturation mapping data presented in the following section.

**Denaturation Mapping**

To perform denaturation mapping, the CLIC device was equipped with a heater as shown in Fig. S6d. A cartridge heater (Omega CSH-101100/120V) was installed in a 13-mm diameter aluminum tube. A 6-mm-diameter fused silica push-lens (Thorlabs LA4966) was glued to the end of the tube. A type T thermocouple (Omega STC-TT-T40-36) was installed with the tip located between the push-lens (~2 mm from the center of the lens) and the upper coverslip. A 0.25-inch-thick G10 thermal insulator was installed between the aluminum tube and the piezo actuator used to form the CLIC chamber. The heater was driven from a variable direct current supply, allowing its power to be adjusted from 0 to 6 W. Denaturation mapping was performed at 42 °C.

To better understand the thermal profile of the system, we performed a simulation using COMSOL (www.comsol.com) Multiphysics 4.3b. We modeled the heated pusher assembly, flow cell, immersion oil between the push-lens and flow cell, objective immersion water, and sample mounting plate (Fig. S6C). The top of the push-lens was held at a fixed temperature of 42 °C and the sides of the aluminum sample plate and the bottom of the immersion water were held at a fixed temperature of 20 °C. The rest of the surfaces were given thermally insulating boundary conditions. The mesh constructed to model the system had a longest linear element of length 1.2 mm on the flow cell holder, and finest element of length 12 µm at the center of the flow cell. The flow cell and the lens were assumed to be made of fused silica and the sample plate, and the heated push-lens mounting tube of aluminum. The sample was modeled as being water. The solver was set to find a steady-state solution of the heat equation with the push-lens in contact with the flow cell.

The simulation results show that the temperature profile across the region of interest varies by less than 0.1 °C (Fig. S6B). λ-phage DNA melting is not sensitive to variations this small. Heating the pusher to 42 °C resulted in a sample temperature of 38 °C. A time-dependent simulation showed that the system took ~30 s to reach a steady-state temperature profile.

**Confinement Theory**

Experimentally, it appears that the DNA molecules are indeed trapped in the nanochannels (Fig. 4): We did not observe deflections or escape of the molecules out of the channels for chamber heights $h$ below 120 nm. We measured the widths of the point-spread functions of the confined molecules and saw no significant change in the chamber height. Consequently, the height of the slit between the two coverslips does not need to be considered explicitly, and can instead be subsumed into the depth of the nanochannel, $D_r$. The problem of nanogroove confinement can be thus reduced to confinement of DNA in a nanochannel occupying a $D_r \times D_r$ cross-section with $D_r = D + h$.

The majority of our DNA extension data falls in a transition regime bordering the strongly confined Odijk limit. This regime, although often characterized by a ~1/D scaling of free energy with channel width $D$, is not well understood (4). In particular, there has been almost no work characterizing DNA stretching in nanochannels with nonunity aspect ratios close to the Odijk limit. [It is known, however, that in this limit the geometric average of the channel dimensions no longer can be used to characterize the stretching (6)]. From an experimental point of view, this regime is doubly challenging in that no analytic results exist to describe measurements straddling the edge of the Odijk/transition regime.

Recently, however, Dai et al. developed a theoretical approach (5) that provides a more convenient description of the transition regime for purposes of comparison with experimental data. Dai et al. argue that in the transition regime, the departure from Odijk behavior can be understood by the nucleation of S-loop structures in the molecule midsection and C-loop structures at the molecule ends. The C- and S-loop statistics can be described via a classic Ising-type of formalism that yields a relation for the extension for $D < 2P$ (given appropriate specification of loop
formation energies). In their model, the nucleation energy for forming a bend $F_u$ is determined by

$$F_u = c_1 P D_y - c_2.$$  \[S4]\n
Parameters $c_1$ and $c_2$ are numerical constants determined from Monte Carlo simulation and are found in ref. 5. These constants depend very weakly on the polymer effective width $w$, which we have taken as equal to 10 nm. Further modifications of this theory could be introduced on account DNA–wall interactions, which are not well understood on the nanoscale dimensions of the presented experimental data, and would enter in a more complicated fashion than as a simple offset (4).

Whereas Dai et al. work with aspect-ratio unity channels (5), we argue that the bending penalty for anisotropic channels can be approximated by arguing that the polymer will preferentially bend along the largest channel dimension, so the energy barrier for bending in anisotropic channels will be set by the largest dimension $D_y$. As excluded-volume interactions are penalized within a loop, there is an additional energy cost,

$$F_s = \frac{k_B T}{6w}.$$ \[S5]\n
Again, Eq. S5 assumes the polymer will preferentially bend along $D_y$. Parameter $c_3$ is another constant determined by Dai et al. (5) from comparison with simulation data.

The curve of extension versus $h$ is then obtained via equations 3, 12, and 14 from ref. 5 with the loop formation energies determined by Eqs. S4 and S5 specified here. To summarize, the chain extension $r$ is given by

$$r = \left(1 - \frac{2}{L} + \frac{4}{L} \frac{r_{\text{Odijk}}}{L} \right) L + 2 L \frac{r_{\text{Odijk}}}{L}.$$ \[S6]\n
The quantity $r_{\text{Odijk}}/L$ is the extension of the chain in the presence of S loops only:

$$\frac{r_{\text{Odijk}}}{L} = r_{\text{Odijk}} \left(1 - \frac{2F_u}{3k_B T} - \frac{\pi D_y f_s}{3}\right).$$ \[S7]\n
The quantities $f_s$ and $L_s$ in Eq. S7 are respectively the average number of S loops per contour length and the average contour length stored per S loop as obtained from solution of the 1D Ising model (equations 6 and 7 in ref. 5). The quantity $r_{\text{Odijk}}/L$ is the Odijk-limit extension of the chain,

$$\frac{r_{\text{Odijk}}}{L} = \left(1 - 0.09137 \frac{D_y^2 + D_y^3}{P^2 L^3}\right).$$ \[S8]\n
with $L$ being the contour length of the entire chain. The quantity $r_{\text{Odijk}}/L$ in Eq. S6 is the C-loop extension:

$$\frac{r_{\text{Odijk}}}{L} = \frac{\exp \left(-\frac{F_u}{3k_B T}\right)}{1 + \exp \left(-\frac{F_u}{3k_B T}\right)}. $$ \[S9]\n
Finally, $L_c$ in Eq. S6 is the contour stored in the average C loop

$$L_c = \pi D_y \frac{\exp \left(-\frac{F_u}{k_B T}\right)}{1 + \exp \left(-\frac{F_u}{k_B T}\right) + 2 \left(1 - \exp \left(-\frac{F_u}{3k_B T}\right)\right) \left(1 + \exp \left(-\frac{F_u}{3k_B T}\right) \right) - \exp \left(-\frac{F_u}{3k_B T}\right)}.$$ \[S10]\n
The theory developed in this work is in good agreement with the maximal measured DNA extension presented in Fig. 4. Future work could extend the theoretical model for DNA molecules within the convex lens-induced nanochannel templating device to include DNA–wall interactions and to describe the transition from strong to weak confinement. Although this work focuses on the confinement regime in which DNA molecules are observed to fully enter nanoscale topographies (chamber heights $h < 100$ nm), future work could explore the behavior of DNA molecules at larger chamber heights (e.g., partitioning of molecules between multiple channels when the imposed confinement is relaxed).

Fig. S1.  Chamber imaging and characterization. (A) Fluorescence intensity of AF647, proportional to the local chamber height, is imaged over a wide field. A grid of images is obtained using a raster scan of the microscope stage, using the methodology developed in ref. 3. Microchannels are visible, but nanochannels are not. (B) Interferometry scan of the chamber. Green contours superimposed upon the first two destructive interference minima correspond to chamber heights of 260 and 520 nm. The region interior to the red circle was used to perform the chamber height fit (radius of 400 μm). The orange cutlines passing through the contact point were used in the presented cross-sectional fits of the chamber height profile. (C) The blue dots and cyan line delineate the AF647 fluorescence intensity as a function of height and respective fit, taken along the horizontal cutline (averaged over a 20-μm thickness). Statistical error bars representing the SE on the mean are smaller than the dots and do not appear.

Fig. S2.  (A) Chamber height cross-sections, corresponding to the horizontal (solid) and vertical (dashed) cutlines in Fig. S1B differ in radii of curvature by 4.9%. (B) Horizontal cross-section of chamber height, corresponding to ten repeated chamber formations. The average radius of curvature of the chamber is $R_{c,\text{avg}} = 129 \pm 6$ mm, where the stated uncertainty is the SD of $R_c$.

Fig. S3.  DNA length determination procedure. (A) Representative single-pixel cross-section of DNA fluorescence intensity (blue) and fit (red), averaged over 10 frames. This fit yields a DNA extension of $16.8 \pm 0.2 \text{ μm}$. The error bars correspond to the standard error on the mean of the plotted intensity. (B) Time-averaged (10 frames) fluorescence image of the DNA molecule. The fitted end points are delineated by red dots. A spatial median filter (3 × 3-square pixel region) has been applied to the time-averaged image for display, but is not used in quantitative analysis. (C) The 2D fit of the DNA fluorescence intensity, also averaged over 10 frames, is shown on the same intensity scale.
Fig. S4. Characterizing the confinement gradient. (A) Schematic of the imaging chamber geometry showing the height gradient across an extended DNA molecule. (B) Difference in chamber height along extended molecules as a function their \( y \) coordinate relative to contact point. Each data point corresponds to a DNA molecule in Fig. 4. (C) Change in height across extended molecules divided by their length, as a function of their \( y \) coordinate. DNA molecules of all orientations are shown, although molecules transverse to the confinement gradient can be expected to show smaller values of \( \Delta h \).
Fig. S5.  (A) Theoretical fractional extension as a function of $h$. Simulations were performed to obtain an extended version of the Odijk theory which includes S and C loop as well as the CLIC imaging geometry, as described in Confinement Theory. (B) Variation of fractional extension, $\Delta \varepsilon$, calculated for each molecule in Fig. 4 and Fig. S4 inside the region of validity of the theory, using the $r(h)$ relation in A and the corresponding value for $\Delta h$. (C) The corresponding value of $\Delta h$ is shown on the same scale for clarity. As with Fig. S4, DNA of all orientations are shown.

Fig. S6.  (A) Schematic of the lens holder with heater embedded. It is thermally insulated from the rest of the CLIC device by a G10 spacer and the temperature is measured by a thermocouple close to the push-lens. (B) Simulated temperature profile inside the flow cell, centered at the contact point. This shows that the temperature is uniform within the imaging region of interest to a few hundredths of a degree. (C) The simulation model showing the temperature profile.
**Movie S1.** Fluorescence movies of loading DNA molecules into nanochannels, as described in Fig. 3.

**Movie S1**

**Movie S2.** Fluorescence movies of templated DNA molecules, as described in Fig. 4.

**Movie S2**
Movie S3. Fluorescence movies of loading DNA molecules into nanopits, as described in Fig. 5A.

Movie S4. Fluorescence movies of DNA molecules in nanopits, as described in Fig. 5B.