Miniaturized flow cell with pneumatically-actuated vertical nanoconfinement for single-molecule imaging and manipulation

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Convex Lens-induced Confinement (CLiC) is a single-molecule imaging technique that uses a deformable glass flow cell to gently trap, manipulate, and visualize single molecules within micro- and nano-structures, to enable a wide range of applications. Here, we miniaturize the CLiC flow cell, from $25 \times 25$ to $3 \times 3 \text{ mm}^2$ and introduce pneumatic control of the confinement. Miniaturization of the flow cell improves fabrication throughput by almost two orders of magnitude and, advantageous for pharmaceutical and diagnostic applications where samples are precious, significantly lowers the internal volume from microliters to nanoliters. Pneumatic control of the device reduces the confinement gradient and improves mechanical stability, while maintaining low autofluorescence and refractive index-matching with oil-immersion objectives. To demonstrate our “mini CLiC” system, we confine and image DNA in sub-50 nm nanogrooves, with high DNA extension consistent with the Odijk confinement regime.

I. MOTIVATION

A. Single-molecule imaging

Simple and user-friendly single-molecule imaging tools are needed to advance a wide range of fields, including molecular biology, diagnostics, nanomaterials, and pharmaceuticals. Bulk measurements on samples often gloss over biologically relevant information which is hidden in the noise by ensemble averaging over many molecules. For instance, single-molecule measurements can detect the rare events and outliers within largely heterogeneous populations that are often associated with the onset of diseases such as cancer. To an increasing degree, single-molecule measurements and their advantages are being established and recognized in the research sector. A wide range of opportunities to apply these methods to solve real-world problems in the biotechnology and health sectors are opening up, with exciting research and development challenges ahead.

Imaging single biomolecules in free solution, with high signal-to-noise, and for long observation times, has remained challenging due to background fluorescence from out-of-focus molecules and drift of molecules in and out of the microscope’s thin focal plane. Current single-molecule fluorescence techniques face limitations in observation times and sample concentrations, and remain cumbersome to implement. For example, total internal reflection fluorescence (TIRF) microscopy eliminates out-of-focus background, and is typically implemented by tethering one molecular species to a surface for prolonged observation, while a second species diffuses in solution. The $\sim 100 \text{ nm}$ thickness of the imaging volume, although thinner than the microscope’s focal plane, limits the maximum sample concentration to $\sim 100 \text{ nM}$ or lower. TIRF typically achieves observation times in the millisecond range or longer for single diffusing molecules, as they drift in and out of the microscope focal plane.

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A second standard fluorescence microscopy technique is confocal microscopy, where molecules are observed as they diffuse in and out of a focused “laser spot”, corresponding to the diffraction-limited excitation volume\textsuperscript{12–14}. Confocal microscopy achieves high signal-to-noise at the expense of very short, typically sub-millisecond, observation times. Concentrations are typically limited to \( \sim 10 \) nM to ensure that only one sample molecule is present in the excitation volume at a time\textsuperscript{12}. A third standard technique is optical tweezers, which hold molecules in position and thus enable long observations, but require that molecules be tethered and thus introduce bias on their available shapes and configurations\textsuperscript{14}.

In this work, we introduce pneumatic confinement control and a miniature implementation of the Convex Lens-induced Confinement (CLiC) imaging method – a method which we have pioneered to overcome the challenges of concentration, observation period, and tethering. By passively confining molecules within nanoscale features, CLiC can enable single-molecule observations up to several hours or more, at concentrations up to the micromolar range\textsuperscript{12,15}, and under closer to native conditions.

B. Convex Lens-induced Confinement Imaging

In CLiC imaging, molecules are confined between two glass surfaces, separated by a thin spacer, that are pressed into contact with each other by deflecting a flexible top surface into a more rigid bottom surface. The distance between the surfaces once deflected increases gradually away from the point of contact, and molecules in the region surrounding this point are confined within a nanoscale gap thinner than the objective’s focal plane. In this region, there is no background contribution from out-of-focus fluorescent molecules, and confined molecules can be imaged and tracked for minutes to hours\textsuperscript{12,15,16}.

Small structures, such as pits\textsuperscript{17,18} or grooves\textsuperscript{16,17}, can be etched into one or both of the glass confining surfaces. Individual molecules are trapped within these structures as the surfaces are pressed together, allowing for long observation times limited principally by sample stability and photobleaching. Molecules very close to the point of contact can be completely sealed within the structures, while molecules further out are entropically trapped until the gap height becomes comparable to the size of the molecules.

The first CLiC instruments achieved nanoscale confinement by pressing a convex lens against a coverslip on which a drop of sample solution was held\textsuperscript{12}. The curvature of the lens forms a smoothly-varying nanoscale gap between the surfaces surrounding the point of contact. The lens was actuated manually using a micrometer to control the confinement. The use of a deformable glass flow cell to contain the sample was later introduced to prevent evaporation of the sample, reduce oxygen exposure, and enable control of fluid flow\textsuperscript{3}. In the flow cell implementation of CLiC, the convex lens presses the top surface of the deformable flow cell against the bottom surface, forming a nanoscale gap between the flow cell surfaces surrounding the point of contact. In the most recent CLiC implementations\textsuperscript{15,16}, precisely-controlled nanoscale confinement is achieved using a piezoelectrically-actuated nanopositioner to deflect the flow cell top surface. The flow cell is constructed from two 25 × 25 mm\textsuperscript{2} coverslips bonded with \( \sim 10-30 \) µm thick double-sided adhesive tape that has a wide flow channel laser-cut through it. The flow channel is approximately circular in the center with a diameter of 15 mm. These dimensions give a very gradual chamber curvature when the top surface is deflected into contact with the bottom surface. The nanopositioner motion range (typically 100 µm), can fully deflect the flow cell with sub-nanometer positioning resolution. The CLiC instrument mounts on top of any inverted fluorescence microscope, making it easy to implement and suitable for a wide range of applications.

C. Applications of Simple Single-molecule Imaging

CLiC is optimized for solution-based, single-molecule measurements, with observation periods ranging from milliseconds to hours. For example, we recently imaged the binding
of fluorescently-labeled oligonucleotides to unwinding sites on supercoiled DNA plasmids, where both species are freely diffusing but confined within micropits\textsuperscript{18}.

Importantly, since the binding kinetics are driven by internal conformational fluctuations in the plasmids, the tethering required by other methods is undesirable. In the CLiC binding assay, the pits sealed by the top confining surface were shallower than the focal plane, but still much larger than the molecules so as to emulate native confinement conditions\textsuperscript{18}.

By viewing a large array of trapped molecules, we detected and counted rare binding events, as few as one per several hundred, among thousands of copies of molecules. We performed observations over tens of minutes with exposure times of tens of milliseconds, quantifying binding rates on the order of $10^6$ M$^{-1}$s$^{-1}$. Observation times were limited only by photobleaching, which was suppressed by using oxygen scavengers and confining the solution to a sealed glass flow cell. Confining and imaging many copies of molecules within arrays of micro or nanoscale features enables simultaneous 1) accurate readings of kinetic rates and affinities with high statistics and 2) inspection, identification, and removal or further study of individuals and outliers such as aggregated or surface-immobilized molecules.

CLiC also enables loading and linearization of long polymers like DNA from the flow cell working volume and into nanogrooves without the use of electrophoresis. This is compatible with a wide range of buffers and ionic strengths\textsuperscript{17,19–21}, which is important to applications in polymer science, biomedicine, DNA sequencing, and biotechnology. We used linear nanogrooves and an adjustable nanoslit to study the DNA extension as a function of vertical confinement, enabling us to validate and connect polymer theories over a continuum of size scales\textsuperscript{20}.

Loading molecules into circular nanogroove structures, which can be entirely closed, is also enabled by CLiC\textsuperscript{16}. We used circular nanogrooves to trap linearized DNA, prevent confinement gradient-induced drift, and enable reactions which would have little to no yield in bulk – such as end-to-end ligation reactions\textsuperscript{16}.

D. Current challenges in CLiC microscopy

Despite the advantages of CLiC imaging, there are challenges to overcome to fully exploit its potential and bring it to the forefront of important applications requiring high-throughput single-molecule imaging, such as diagnostics and pharmaceuticals. Improving flow cell rigidity, stability, and fabrication throughput are essential to increasing the repeatability of experiments, reducing drift, reducing sample volume, and lowering experiment costs. These improvements have motivated the research and development in this work, in order to enable impactful applications of CLiC imaging.

1. Microfabrication throughput

Most CLiC experiments are performed using flow cells patterned with arrays of microscopic structures within which single molecules can be confined. Producing these flow cells requires photolithography or nanolithography techniques (such as electron beam lithography) and reactive ion etching in a clean room environment, which is costly due to the large size of the devices and small number of die per wafer. Due to the high cost of producing 25 mm CLiC flow cells, they are typically disassembled, cleaned, and reassembled following an experiment so that they may be used again. This consumes a researcher's time and introduces cumbersome experimental steps in a fumehood, using harsh reagents.

2. Flow cell mechanical rigidity

The current 25 mm flow cell is large enough to accommodate an oil-immersion objective with a standard working distance and a clamping system with fluidic connections that
supports the flow cell. This format leaves sufficient clearance between the clamp and objective for microscope stage travel. Objectives suitable for single-molecule imaging have short working distances and are designed for use with #1.5 coverslips, with a typical thickness of 175 ± 15 µm. To achieve optimal image quality in the CLiC system we construct the flow cell with a #1.5 coverslip as the bottom surface, and a #1 coverslip as the top deflecting surface. Since this makes the flow cell quite thin, deflecting the top confining surface causes the bottom surface to deflect significantly, meaning that the microscope’s focus to be continually adjusted as confinement is varied. The flexibility of the flow cell can also result in some drift over observation times of many minutes. This can be overcome by using an autofocus system, but such systems may fail to operate when performing CLiC experiments with sub-wavelength confinement structures, such as the nanogrooves demonstrated in this work.

3. Temperature stability

We are able to perform experiments at elevated temperatures using a heating system, in which we heat the oil-immersion objective and the aluminum tube on which the CLiC lens is mounted. The temperature changes introduce additional drift, caused by thermal expansion of mechanical components such as the nanopositioner and rod holding the CLiC lens that further deflect the flow cell. This causes the confinement to vary with temperature, particularly at an elevated temperature using a heating system.

4. Sample requirements

The 25 mm CLiC flow cell has an internal volume of about 5 µL. For robust experiments where flow control is required, several times this volume is used. We typically use 20-50 µL, which can present a problem when working with precious protein and DNA samples. Much of the internal volume acts as “dead” volume, making sample replacement and reagent exchange challenging.

II. SOLUTION: MINIATURIZATION OF CLiC PLATFORM

We present a miniaturized CLiC flow cell that overcomes many of the challenges associated with the larger 25 mm format. The flow cell is actuated pneumatically to simplify operation, eliminating the piezoelectric actuator and associated micropositioning stages for manual alignment used in current CLiC instruments (see Fig. 1). The device has improved rigidity and stability due to reduced thickness of the top (but not the bottom) confining surface, elimination of adhesive tape in the flow cell construction, and elimination of the actuating nanopositioner. The flow cell can be actuated repeatably, eliminating the need for manual alignment of a CLiC lens with the confinement features.

In presenting the miniaturized, pneumatic format of CLiC imaging, we begin by describing A.) the device design, fabrication and characterization. We next describe our B.) the fluidic and pneumatic interface, emphasizing physical modeling and quantitatively understanding the consequences of bringing glass walls within close (nanoscale) proximity of one another to achieve C.) pneumatic control of the confinement geometry. Miniaturizing the CLiC platform required modeling and understanding the consequence of multiple forces on flow cell walls, and their behavior on the nanoscale, in order to achieve successful operational parameters.
A. Device design and characterization

The miniature flow cell is a 3 × 3 mm² chip fabricated entirely of glass using standard clean room techniques. The smaller size greatly reduces the cost per flow cell and opens the door to more complex microfabrication. Microscopy-quality cover glass is an ideal material for fluorescence imaging; it has very low autofluorescence, a surface roughness of <0.8 nm, and a refractive index that is optimal for use with oil-immersion objectives to minimize aberrations. The top and bottom glass surfaces are fusion-bonded in a clean room environment to achieve a high-pressure seal and excellent chemical resistance with ultra-clean surfaces.

The device, shown in Fig. 1(a), contains a 2 mm diameter, 500 nm deep nanoslit defined by photolithography and etched with buffered hydrofluoric acid (BHF) solution. These dimensions were chosen carefully to prevent stiction of the surfaces when fully deflected, while maintaining a low confinement gradient. The 50 µm thick top glass membrane is flexible and can be deflected pneumatically above the nanoslit to vary the confinement. Two 20 µm deep microchannels are also etched into the bottom surface and holes are etched through the top surface for sample introduction. These features were defined by photolithography and etched with hydrofluoric acid (HF) solution. The flow cells presented here also have nanogrooves of roughly 40 nm width and 40 nm depth defined by electron-beam lithography and etched into the bottom surface by reactive ion etching (RIE), as shown in Fig. 2(b) and (c). The internal volume of the flow cell is approximately 50 nL.

To load DNA into the nanogrooves, the sample is first introduced into one of the microchannels, and pressure is applied to flow it through the entire length of the microchannel. Pressure (∼25-50 kPa) is applied to the glass membrane during the microchannel loading to prevent the sample from entering into the confinement region near the center of the device (see Fig. 1(b)). Next, suction (∼25-50 kPa) is applied to the membrane to increase the gap height (typically to > 1 µm) enough so that DNA can be flowed into the nanoslit region. This is done by applying pressure to both fluid ports at the ends of the microchannel into which the DNA was initially loaded, driving DNA across the center of the flow cell and into the other microchannel (see Fig. 1(c)). Pressure (∼25-50 kPa) is then applied to the membrane once again to deflect the membrane downwards, causing DNA to enter the nanogrooves and adopt an extended conformation (see Fig. 1(d)). The procedure is the same for other confinement structure geometries, such as pits or circular grooves, while adjusting the pressure to determine the final confinement. Features can be completely sealed by applying excess pressure to flatten the top surface against the bottom, or a nanoscale gap can be maintained to allow for reagent exchange. The two microchannels

![Diagram of flow cell design](image)

FIG. 1. Pneumatically-actuated flow cell design. (a) Labeled photograph of the flow cell chip. (b) Fluid ports and microchannels allow for sample introduction into the chip. Sample is loaded into one of the ports and pressure is applied to fill the microchannel with sample. Pressure is applied to the glass membrane which forms the top surface of the nanoslit to keep the sample confined to the microchannel. (c) Suction is applied to the membrane to raise to raise the nanoslit to a height > 1 µm. Pressure is then applied across the two microchannels to drive the sample into the nanoslit area. (d) Pressure is applied to the glass membrane, causing it to deflect and drive molecules into confinement nanostructures.
can be used to introduce two separate reagents into the flow cell simultaneously, which can then be mixed in the central region by raising the membrane.

Since the flow cell size is comparable to that of the front element of a typical microscope objective, it was not practical to clamp the flow cell on both sides as was done with the 25 mm devices. To support the flow cell mechanically and form fluidic and pneumatic seals, we bonded the chips to small plastic carriers using a thin layer of adhesive, shown in Fig. 2(a). Since there is no adhesive above the deflecting glass membrane, it does not contribute any autofluorescence background during experiments. The carrier contains four fluid reservoirs, each with a capacity of 4 µL, that connect to the four fluid ports on the flow cell. At the beginning of an experiment, the sample to be imaged is first introduced into a reservoir on the plastic carrier and a small pneumatic pressure is applied to the reservoir to drive the sample into the glass flow cell through the etched inlet holes.

![Fabricated flow cell chip](image)

**FIG. 2.** Fabricated flow cell chip. (a) Fabricated chip glued to a custom plastic carrier to facilitate clamping for use with short-working-distance objectives. (b) and (c) Scanning electron micrographs of nanogrooves etched into the glass.

We used atomic force microscopy (AFM) to characterize the flow cell surface roughness. Figure 3 shows AFM images of the glass before and after etching the nanoslit. The root mean square (RMS) roughness before and after etching is 0.46 nm and 4.4 nm, respectively. This was adequate for linearizing DNA in nanogrooves but could be improved, for instance, by modifying the etching solution.

![Atomic force microscopy (AFM)](image)

**FIG. 3.** Atomic force microscopy (AFM) of the glass surface in the nanoslit region before (a) and after (b) etching to 500 nm depth using standard methods\(^1^6\). The etched surface is rougher but is still adequately smooth for DNA linearization experiments.
B. Fluidic and pneumatic interface and instrumentation

The pneumatic and fluidic connections to the flow cell were made by clamping the glued flow cell carrier assembly between a machined aluminum base and a plastic manifold. The base has a 200 μm thick flange that supports the carrier while allowing the flow cell chip to protrude through the base, making the instrument compatible with short-working-distance objectives. Tubing connections to the manifold were made with flat-bottomed compression fittings. A seal between the flow cell chip carrier and manifold is formed by a silicone gasket (see Fig. 4).

![Diagram of fluidic and pneumatic connections]

FIG. 4. Fluidic and pneumatic connections. (a) Exploded view of the instrument, showing the flow cell chip, gasket, and manifold for making fluidic and pneumatic connections to the chip. (b) Photograph of assembled instrument.

C. Achieving pneumatic control of the vertical confinement

In designing the miniature flow cell, we optimized the geometry to avoid spontaneous collapse of the membrane due to attractive Van der Waals forces between the surfaces, while minimizing the curvature of the deflected surface to minimize confinement gradient. The restoring force of the membrane when deflected into contact with the bottom surface must therefore be larger than the Van der Waals force.

Since the device is actuated pneumatically, the glass membrane is deflected by a force applied uniformly over its area, rather than a point force applied only at the center. This results in a reduction of the confinement gradient in the central region of the flow cell compared to the point force case, as shown in Fig. 5(a). The flexural rigidity $D$ of the glass material is defined as $^{24}$:

$$D = \frac{E t^3}{12(1-\nu^2)}$$

where $E = 72.9$ GPa is the Young’s modulus of glass, $\nu = 0.208$ is Poisson’s ratio, and $t$ is the thickness of the deflecting surface. We model the top confining surface as a clamped-edge disk of radius $a$, thickness $t$, and flexural rigidity $D$ as given in Eqn. 1. The bottom
surface is a flat plate separated a fixed distance \( h \) from the top plate. If the surface is deflected by a point force applied to the center, as is done in CLiC by pressing a convex lens into the surface, then the force \( F \) required to deflect the center of the surface a distance \( d \) is given by\(^{25}\):

\[
F = \frac{16\pi Dd}{a^2} \tag{2}
\]

and the distance \( w(r) \) between the top and bottom surfaces of the flow cell, a distance \( r \) from the center is given by the following\(^{25}\) and plotted in Fig. 5.

\[
w(r) = h - d \left( 1 - \frac{r^2}{a^2} + 2 \frac{r^2}{a^2} \ln \left( \frac{r}{a} \right) \right). \tag{3}
\]

![Flow cell deflection curves.](image)

**FIG. 5.** Flow cell deflection curves. (a) Gap height profile as predicted by Eqn. 3 for the 25 mm and 3 mm CLiC flow cells deflected by a point force applied to the center, and by Eqn. 5 for the pneumatically-actuated 3 mm flow cell. (b) Attractive Van der Waals force between the glass surfaces and restoring force of the glass membrane as a function of deflection. The spring force is large enough to prevent stiction due to Van der Waals attraction.

In the case of pneumatic actuation, a uniform pressure is applied to deflect the surface. In this case, the pressure required to deflect the center of the surface a distance \( d \) is given by\(^{25}\):

\[
P = \frac{64Dd}{a^4} \tag{4}
\]

and the distance between the top and bottom flow cell surfaces is given by\(^{25}\):

\[
w(r) = h - d \left( 1 - \frac{r^2}{a^2} \right)^2. \tag{5}
\]

These deflection profiles are shown in Fig. 5(a) for the 25 mm flow cell with plate separation \( h = 20 \) \( \mu \)m and for the 3 mm flow cell with \( h = 0.5 \) \( \mu \)m under both nanopositioner (point force) actuation and pneumatic (uniform force) actuation, for the case of maximum deflection where \( d = h \). Because \( h \) has been greatly reduced for the miniature flow cell, initial loading of molecules into the nanoslit for confinement is done by first raising the deflecting surface using applied suction. A smaller value of \( h \) results in a reduced confinement gradient and increased area where sample molecules are confined.

Equations 3 and 5 ignore plastic deformation of the glass surfaces due to a combination of the applied force and Van der Waals adhesive forces. These forces result in an area
Increasing confinement

Increasing confinement

1 mm
d ≈ -2.2 μm d ≈ -1.4 μm d ≈ -0.5 μm d ≈ 0 μm d ≈ 0.5 μm

FIG. 6. Photographs of a flow cell showing thin film interference fringes in the nanoslit, showing the radially symmetric deflection. The membrane is under suction in the first 3 images from left to right, under no applied pressure in the 4th image, and under positive pressure in the 5th image where the surfaces are in contact at the center. Confinement increases from left to right. The center deflection \( d \) is estimated from the interference pattern using a Michel-Lévy chart\(^2\). The edge separation between the top and bottom glass surfaces is \( h \approx 500 \text{ nm} \).

of contact between the surfaces rather than a single point. This area can be significant, especially when pressures larger than \( 64Dh/a^4 \) are applied in the pneumatic case, and result in the flattening of the top surface against the bottom. This can be used to seal molecules inside confinement structures.

There is some limitation to how small \( h \) can be based on Van der Waals forces between the glass surfaces when they are very close together. The deflecting glass membrane acts as a spring, providing a restoring force that acts against the Van der Waals force to prevent stiction between the surfaces. The Van der Waals force \( F \) between two flat plates separated by a distance \( z_0 \) is given by\(^2\):

\[
\frac{F}{\text{Area}} = \frac{A_H}{6\pi z_0^2}
\]  

(6)

where \( A_H \) is the Hamaker constant, for which we used a value of \( 0.63 \times 10^{-20} \text{ J} \) for fused silica in water\(^2\). When the top surface is curved with the deflection profile \( w(r) \) from Eqn. 5, Eqn. 6 is integrated to give the Van der Waals force between the surfaces for a given deflection \( d \):

\[
F = \frac{A_Ha^2}{48h^3} \left( \frac{h(5h - 3d)}{(h - d)^2} + \frac{3\tanh^{-1}\left( \sqrt{\frac{d}{h}} \right)}{\sqrt{\frac{d}{h}}} \right). 
\]  

(7)

This equation is plotted along with the spring force of the deflecting surface in Fig. 5(b) for \( h = 500 \text{ nm} \) in water. The spring force is larger than the Van der Waals force for gap heights larger than the length of a hydrogen bond, so the chamber does not collapse when the surfaces are brought into contact at the center.

In summary, we chose a diameter of 2 mm for the confinement region, a membrane thickness of 50 μm, and an edge spacing of 500 nm between the confining surfaces as this configuration produces a significantly reduced confinement gradient compared to the 25 mm format, while avoiding spontaneous membrane collapse due to Van der Waals forces. Photographs of a miniaturized flow cell showing a changing Newton’s rings thin-film interference pattern as confinement is increased are shown in Fig. 6. These parameters provided a successful solution to the implementation of “mini CLiC”, achieving the central goal of this work.
Increasing confinement

FIG. 7. Montage of λ DNA loading and linearization in nanogrooves within the miniaturized flow cell. (a) DNA are initially loaded into the nanoslit region with suction applied to the membrane. The pressure is then increased to start increasing the confinement. (b) and (c) Increasing confinement causes the individual DNA molecules to become distinguishable. All molecules are confined within the focal plane of the objective. (d) DNA begin to enter the nanogrooves. Confinement is applied more slowly to ensure gentle loading without DNA breakage. (e) Once maximum confinement is reached, DNA are fully confined and linearized within the nanogrooves, suitable for individual analysis.

III. DEVICE OPERATION AND DEMONSTRATION

Preparing linear DNA molecules in nanochannels is useful for genome mapping\textsuperscript{28–30} as well as polymer physics studies\textsuperscript{31,32} and studies of protein-DNA and other chemical reactions\textsuperscript{21,33,34}. When the channel width and depth are smaller than the 50 nm DNA persistence length, loops in the DNA conformation are suppressed (the Odijk confinement regime)\textsuperscript{35} and the sequence is arranged in order along the channel axis.

Introducing DNA or protein-DNA complexes from bulk solution into nanochannels without breakage can pose a challenge for side-loading nanofluidic technologies due to the large free energy barrier that must be overcome. One solution has been to combine electrophoresis with post arrays to untangle DNA, and with “funnels” to gradually transition the DNA towards lower entropy conformations\textsuperscript{36}.

An alternative nanogroove-loading strategy which we have developed – to load DNA into open-face nanogrooves from the top using CLiC\textsuperscript{17} – provides the most stringent test of the miniature-format CLiC device. Top-loading reduces the free-energy barrier and is inherently gentle, as the confinement is increased gradually, and results in no DNA breakage. We previously characterized DNA extension in $27 \times 27$ nm\textsuperscript{2} nanogrooves as a function of applied confinement\textsuperscript{17}.

In this work, we used the miniaturized flow cell to load and extend λ-phage DNA in linear nanogrooves via top-loading to demonstrate its operation and ability to attain high confinement. Narrow nanogrooves are a good test of the device operation, as loading DNA into them requires clean surfaces, low surface roughness, and fine control of the confinement.

A. Mini-CLiC imaging results: DNA in nanochannels

A montage of the DNA linearization process is shown in Fig. 7. DNA molecules were first introduced into the nanoslit with suction applied to the membrane, and the pressure was gradually increased over $\sim10$-20 seconds to gently load the DNA into the nanogrooves. We observed up to $\sim85\%$ extension, which is expected for confinement in the Odijk regime but may have been somewhat limited by the roughness of the etched bottom surface. This extension was observed across a large region due to the low confinement gradient. The sample remains in focus as confinement is applied, and no focus correction is required during the loading process. This experiment is a good test of device performance, as it requires a high degree of confinement to reach the Odijk regime\textsuperscript{17}. 
B. Methods

1. Sample preparation

The λ DNA sample was prepared at a concentration of 50 ng/µL in 0.5X TBE buffer (45 mM Tris, 45 mM boric acid, and 1 mM EDTA) with 5% polyvinylpyrrolidone (PVP) added to reduce non-specific binding of DNA to the glass surfaces, as in prior work\textsuperscript{15}. We inserted 3 µL of the sample into one of the reservoirs in the plastic flow cell carrier for the experiment.

2. DNA loading and confinement

The flow cell assembly (with the attached carrier) was first inserted into the aluminum clamp, and mounted on the microscope. The DNA sample was then introduced into one of the reservoirs on the flow cell chip carrier using a pipette. The fluidic manifold was mounted on top of the flow cell and clamped down with screws to form a seal.

Air pressure was applied from a syringe to propel the sample into one of the microchannels on the flow cell. Suction was then applied to the flexible membrane using a syringe to increase the height of the nanoslit so that the sample could be driven into it by pressure applied to both ends of the microchannel. We then applied pressure to the membrane to increase the vertical confinement in the nanoslit and drive the DNA into the nanogrooves, where they adopt a linear confirmation. Since the flow cell requires \( \sim 25 \) kPa for full compression, fine control of the deflection and loading process can be obtained simply using a handheld syringe.

3. Imaging parameters

Imaging parameters were similar to previous CLiC nanogroove experiments\textsuperscript{16,17}. YOYO-1-labeled DNA molecules were excited with a 488 nm semiconductor laser (Coherent Sapphire) and imaged with a 100× NA 1.49 CFI Apo-TIRF objective (Nikon) on a Nikon Ti-E microscope equipped with a cooled electron-multiplying CCD (EMCCD) camera (Andor iXon Ultra) acquiring frames at 50 ms exposure time with an electron multiplying (EM) gain of 300. This produces good image quality while resolving DNA length fluctuations within the nanogrooves. Data acquisition and microscope control was performed in Micro-manager using custom add-on modules, as in our prior work\textsuperscript{15}.

IV. CONCLUSIONS AND OUTLOOK

The device presented here builds on the current CLiC technology, improving the stability and confinement gradient while greatly reducing the device size and simplifying the actuation mechanism. These advancements are important for advancing research applications such as protein-DNA interaction studies, diagnostics, and drug discovery and development. We demonstrated the device’s operation by linearizing λ DNA in nanogrooves, achieving high stretching within the Odijk confinement regime, which is the most stringent available test of the CLiC technique.

The chamber geometry is formed with greater repeatability and predictability, and is more stable due to the increased device rigidity. We expect that these improvements will enable CLiC experiments with a higher degree of reproducibility and detailed control, eliminating manual alignment and refocusing procedures that are necessary with the larger piezo-actuated instrument.

Fabrication throughput has been greatly increased due to the reduced flow cell size. In this work we fabricated flow cells on 25 × 25 mm\(^2\) substrates, yielding 36 devices each.
Future devices with a slightly modified design will be fabricated on 100 mm wafers, which we expect will yield \( \sim 360 \) devices each.

The flow cell internal volume has been reduced from \( \sim 5 \) µL to \(< 50\) nL, a critical improvement when working with precious biological samples due to the high concentrations typically used in CLiC experiments. This improvement alone opens the door to new applications which use precious biological samples.

Further, the use of pneumatic actuation allows the top surface to be pressed against the bottom surface such that an area of contact that increases with applied pressure is formed between them. This can seal molecules within large numbers of confinement structures, trapping them for indefinite periods.

In building on this work, we will add post stand-offs to one of the flow-cell surfaces so that nanoslits can be formed with a fixed height over a large area. This enables small molecule reagents to be perfused over entrapped macromolecules, which are confined entropically in micropits or circular nanogrooves. We will apply this “reaction visualization” platform to enabling new single-molecule studies of binding kinetics; in particular, to develop applications in diagnostics and pharmaceutical sciences which are limited to using ultralow sample volumes. In addition, we will further characterize the achieved deflection profiles when “excess” pressure is applied to seal molecules in large numbers of confinement structures. We conclude this manuscript with a sense of excitement, as this miniature implementation of CLiC will broaden the spectrum of single-molecule microscopy and application development across a wide range of fields.

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