BIOLOGICAL CONFINEMENT PHYSICS: SQUEEZING NEW INFORMATION OUT OF COMPLEX MACROMOLECULES

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By confining and visualizing biomolecules on molecular to microscopic dimensions, we can directly investigate how confinement physics can regulate macromolecular behaviour. New tools enable us to tackle open biological questions on how physiologically important molecules, such as chromosomal DNA or proteins, behave in nanoscale environments such as the nucleus or the ribosome respectively. Despite strong interest, getting into this "*room at the bottom*" has presented challenges to existing tools. Here, we review a suite of single-molecule methods and experiments that we have devised to study how biomolecules respond to incrementally smaller spaces of applied confinement, as well as their own topology, emulating naturally occuring nano confinement structures; both behaviour and topology can be controlled continuously, and with facility by our methods. The overarching vision of our research is to investigate the complex behaviour of biomolecules, such as protein, DNA, and RNA, with respect to the principle of applied confinement.

INTRODUCTION

Why is the development of new approaches to visualize and manipulate *the behavior of single biomolecules*, *on molecular to microscopic dimensions*, so important to the frontiers of research in biological physics, biotechnology, biology, biochemistry, and nanoscience? It is for two principal reasons: firstly, biomolecules must function in wet, squishy, and crowded cellular environments [1,2]; and secondly, "next-generation" genomics and proteomics technologies can leverage new insights and techniques to advance biomedicine [3]. To this effect, theoretical physicist Henrik Flyvbjerg wrote a commentary in *P.N.A.S.* on our pioneering efforts in single-molecule technology, which he called "*Getting into that room at the bottom*" [4]. Dr. Flyvberg was referring to Nobel Laureate Richard Feynman's visionary 1959 lecture, entitled "*There's plenty of room at the bottom*", in which Feynman predicted a world of invention and discovery of tiny machines and devices, on the order of a billionth of a meter in size, the so-called "nano world". Today, many of these inventions have fundamentally changed our lives, ranging from new nanomaterials to miniature biomedical sensors, which have improved medical treatments, and have provided a growing context for pioneering single-molecule technologies. Dr. Flyvbjerg describes the unique nano manipulation and imaging tools that we are developing as an *important solution to the general problem of controlling and understanding biomolecules "at the bottom" of the length scale*, from Angstroms to micrometers; that is, being able to continuously visualize and hence establish the functional mechanisms of the "nano world", at the intellectual frontiers of materials science, engineering, biology and biomedicine.

SUMMARY

We study how confinement physics regulates macromolecular behaviour, from nano to microscopic dimensions. New tools enable us to tackle open questions on how physiologically important biopolymers behave in nanoscale environments.

Building on this context, this *P.i.C* article reviews our recent contributions to "getting to the bottom" of understanding DNA molecules – the complex biopolymers which encode our genetic makeup – from three perspectives: as a coiled polymer with fascinating physical properties under nano confinement, relevant to understanding the dense interior of the cellular nucleus; as a sequence of bases which stores our genetic code, relevant to understanding evolution and disease; and as a dynamic, structural scaffold which regulates essential cellular processes, relevant to understanding DNA replication, repair and gene transcription.

In line with these overarching perspectives, we review our single-molecule investigations of how DNA conformations and dynamics become qualitatively different when squeezed into nanoscale spaces. Building on this fundamental understanding, we apply the tools we are creating to contribute to the development of a "next generation" of "single-molecule biomedicine" – where the properties of individual protein or DNA molecules can be critical to understanding and detecting disease states. We explore how continuously adjusting the applied confinement

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enables us to literally "unscroll" tangled "DNA-blob" conformations into more regular "DNA-rod" conformations, which are easier to interrogate. By developing gentle, high-throughput approaches to visualizing and manipulating macromolecules, we can contribute not only to biological confinement physics, but also to the advancement of biomedicine.

METHOD

We design and build *de-novo* instrumentation to acquire continuous and high-quality movies of the dynamics and shapes of biomolecules, on adjustable nanoscale dimensions. Building upon our published principle of *Convex Lens-induced Confinement* (CLiC) imaging [5,6], we have developed a microfluidic imaging tool, which mounts on a regular inverted microscope, transforming it into a single-molecule manipulation and visualization station [7]. For example, by confining long, linear DNA molecules within ring-shaped traps (Fig. 1d), we can dramatically enhance the efficiency of their "self-ligation" into loops.

Why is "CLiC imaging" important to advancing biophysics?

The state-of-the-art of detecting macromolecular assembly and dynamics primarily comprises methods to watch single molecules bound to each other, to surfaces created by other macromolecules, or to polymers of the cellular cytoskeleton such as DNA or microtubules, respectively. Commonly used methods, such as "total internal reflection fluorescence" (TIRF) microscopy, allow detection of the appearance and disappearance of individual molecules on or near to a surface. What CLiC provides that TIRF does not allow is the capacity to continuously follow the trajectory of a freely diffusing single molecule for long periods, before it is bound to a substrate, when it is bound and when it dissociates. This capability is particularly important in biophysics because many elements of cellular activity, such as DNA transcription or movement of molecules on microtubules are processive; that is, individual events are dependent on previous events. This memory can often be inferred, but not directly measured with current techniques; but while tracking dynamics with CLiC it now becomes possible to measure. Thus, it is possible that CLiC will contribute to verifying or disproving some of the most fundamental models of biomolecular dynamics, such as DNA-topology-mediated regulation of transcription. Furthermore, by looking at molecules in a thin volume, compared to other techniques, CLiC microscopy can resolve single-molecule trajectories in the presence of orders of magnitude higher reagent concentrations, and over orders of magnitude longer periods, allowing us to discern and follow weak and slow interactions – which is relevant to a wide variety of physiological processes.

For example, in an ongoing collaboration with biochemist Dr. David Levens (NIH Cancer Center) and mathematician Dr. Craig Benham (UC Davis Genome Center), we are studying how a topological property of DNA called "supercoiling" influences the interactions between specific probe molecules and specific unwinding sites on the supercoiled DNA. By trapping the molecules using CLiC, and

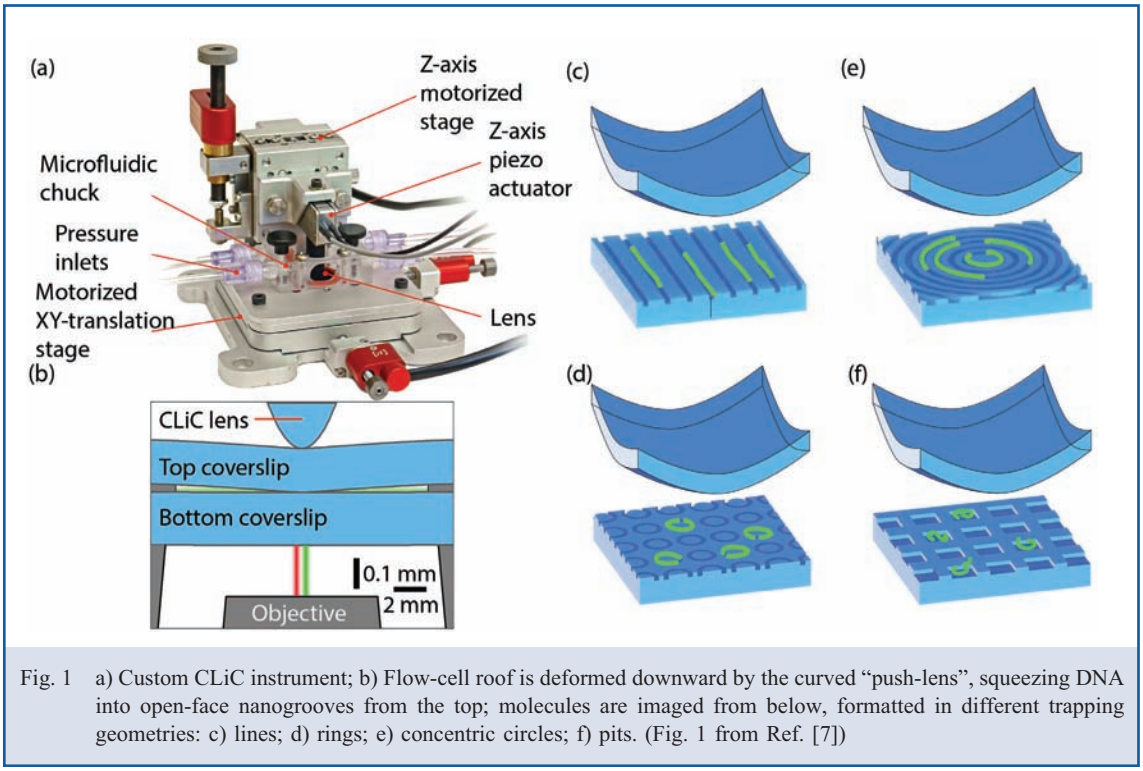

Fig. 1 a) Custom CLiC instrument; b) Flow-cell roof is deformed downward by the curved "push-lens", squeezing DNA into open-face nanogrooves from the top; molecules are imaged from below, formatted in different trapping geometries: c) lines; d) rings; e) concentric circles; f) pits. (Fig. 1 from Ref. [7])

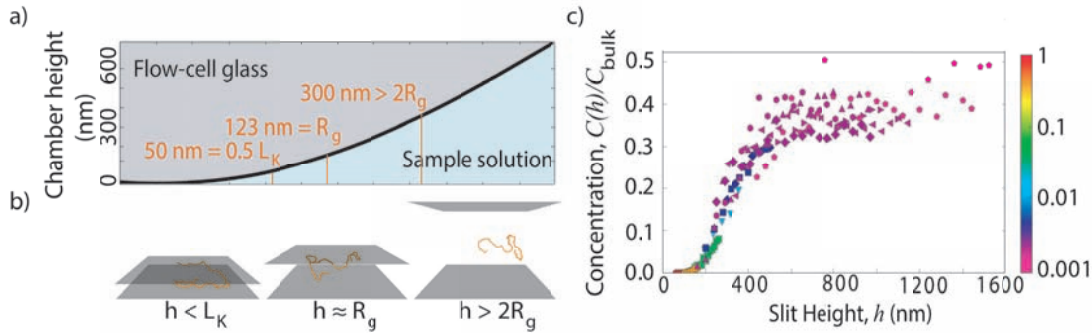

Fig. 2 a) Schematic of a static CLiC imaging chamber (where the flow-cell has been deformed by the push-lens). b) Renderings of different polymer conformations in different confinement regimes obtained by simulation. c) Scaled concentration of the DNA molecules as a function of the confinement height *h*. (Fig. 2 from Ref. [8])

following molecular interaction trajectories over thousands of seconds, we can determine how the binding/unbinding kinetics are influenced by the DNA topology. These insights are important to testing models of gene regulation at a single-molecule level, which were previously inaccessible with other methods under "untethered" molecular conditions.

RESULTS

We are exploring transitions in polymer conformations on nanoscale dimensions

CLiC technology allows us to perform rigorous equilibrium studies of transitions in DNA conformations, e.g., from "blob-like" in an unconfined regime, to "rod-like" in a nanoconfined regime (Fig. 2) [8]. Importantly, we can visualize DNA over several orders of magnitude of applied confinement, in a single experiment and device.

Our research contributes a direct measurement of the "free energy of confinement" experienced by the DNA molecules, over several orders of magnitude of applied confinement. When a flexible polymer is confined, fewer conformations are accessible, making confinement energetically unfavourable. Our measurement of the confinement free energy is direct: we count the number of DNA molecules, at equilibrium, in a single chamber of smoothly varying height. A much lower density of molecules is observed in regions of the chamber where the height is much smaller than the diameter of unconstrained molecules (Fig. 2c).

Importantly, our data provide a "bridge" that connects prior measurements and theories in the literature, typically obtained in limiting cases using devices of fixed dimensions. Our experimental data and simulations have served to link a), "nano confinement theory" (by Odijk [9]), b), an interpolation model which extends Odijk to "moderate confinement" (by Chen and Sullivan [10]) and c), "unconfined theory" which describes the transition from moderate to unconfined conditions (by Casassa [11]). In the nanoconfined regime, the applied confinement is so severe that the polymer is unable to form random coils the way it does in free solution; its conformations look like "deflecting stiff segments" off of the walls. In the unconfined regime, the polymer appears as a "blob", as one would intuitively expect. In between – in moderate confinement – the DNA conformations smoothly transition from a deflecting rod, to a network of blobs, to a single blob, according to theory that our experiments and simulations agree with.

We can dynamically manipulate DNA conformations with *in-situ* visualization

Here, we leverage our fundamental understanding of, and physical control over DNA conformations, to study the *dynamic manipulation* of DNA molecules [4]. We use the curved surface of the "push-lens" (Fig. 1a) to dynamically deform the top flexible coverslip above the bottom coverslip, which contains the embedded nanogrooves, during the CLiC imaging experiment. This work establishes a novel method for *entropic trapping and dynamic stretching* of DNA molecules in grooves, which is compatible with single-molecule, "long-read" genomic analysis. In particular, we can extend DNA in sub 50-nm nano-channels with high and uniform stretching (90%), in agreement with the Odijk deflection theory, and useful towards "long-read" sequence measurements [12,13].

How is nanoscale DNA confinement physics relevant to advancing genomics?

Modern medicine has entered the genomic age; the promise of genomic medicine is to customize optimal treatments for individuals and genetically similar people. Human chromosomes are between 25 million and 425 million bases long; however today's commonly used, high-accuracy DNA sequencing techniques can generally read DNA molecules that are 250 bases long or shorter. Typically, they break chromosomes into random, short and overlapping fragments,

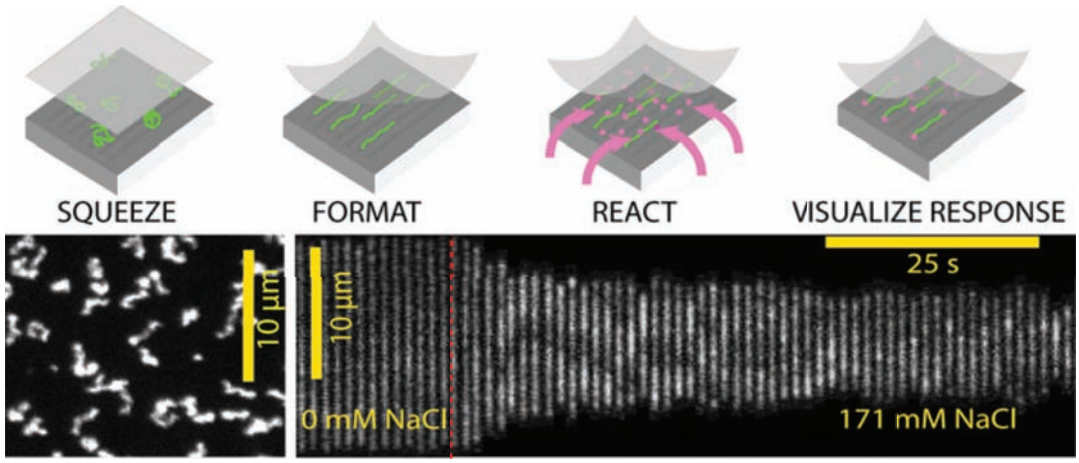

Fig. 3 (Top) Schematic process of squeezing and linearizing DNA molecules into nanogrooves, reaction with reagents, and visualizing the DNA response. (Bottom, left) Image of DNA being squeezed; (right) Kymograph of a single DNA in a nanogroove, whose extension is decreasing over time, due to the exchange of the buffer with a higher salt solution. ("Thumbnail" of Ref. [14])

sequence them, and then match overlapping sequences to each other to reassemble the entire sequence, like pieces of a puzzle. While computational approaches can put more than half of the puzzle back together, this has not worked for critical regions of the human genome containing repeats of the same sequences, such as the genes that code for antibodies, the molecules that protect us from invading bacteria and viruses. To contribute to the growing field of "long-read" genomics, we are continuing to advance our understanding of, and control over DNA in nano-confined spaces, while miniaturizing our tools into a "technology-compatible" format.

We can control and visualize biophysical chemistry in nano confined reactors

Our recent work has demonstrated a new approach to 1) trapping and formatting the shapes of DNA within custom-shaped (e.g., linear or circular) embedded grooves; and 2) introducing reagents and visualizing interactions, without disrupting the molecules [14]. For instance, we can enhance the "joining" of the ends of linear DNA, which are confined in circular nanogrooves, to form closed circular DNA loops, by introducing "ligase enzymes" into the chamber from the side. During these processes the DNA conformations are only limited by the walls of their containers; that is, not tethered.

OUTLOOK

A "tether-free", single-molecule playground for squeezing and visualizing macromolecules, on nanoscale dimensions

Acquiring continuous movies of the dynamic change in biomolecular properties, in response to biochemical and physical variables, enables direct investigation of important biophysical mechanisms. In collaboration with biologists, biochemists, chemists and biophysicists, we aim to apply CLiC technology to investigate a myriad of confinement-mediated processes in the near term, such as protein condensation, protein folding, membrane biophysics and DNA nanostructure dynamics. With the advancement of sophisticated nanolithography tools running in parallel with our research, we are excited by the promise of carving increasingly complex physiological architectures out of glass and silicon walls; allowing us to isolate, watch and understand complex biological molecules at work, in an ever-expanding *room at the bottom*.

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