

Ordered Arrays of Native Chromatin Molecules for High-Resolution Imaging and Analysis

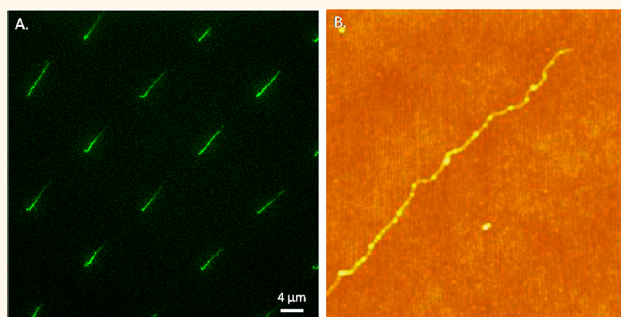
Aline Cerf, Harvey C. Tian, and Harold G. Craighead*

School of Applied and Engineering Physics, Cornell University, Ithaca, New York 14853, United States

Chromatin consists of repeating nucleosome units that contain two pairs of four types of histone proteins (H2A, H2B, H3, and H4) forming an octamer or eight-unit histone core that is wrapped 1.65 turns by a 147 base length of DNA controlling access to the underlying sequence. The composition, modification, and structure of chromatin play a crucial role in gene expression. In mammalian organisms, epigenetic gene regulation functions through methylation of CpG dinucleotides and remodeling of chromatin structure through post-translational histone modifications such as acetylation, methylation, ubiquitylation, poly(ADP)ribosylation, and phosphorylation that can affect the biophysical properties and signaling of regulatory factors within the chromatin template. In the study of a variety of diseases, there is an active effort to map genome-wide genetic and epigenetic patterns across cell types, the latter in response to various environmental influences^{1,2} for the understanding of gene regulation and for medical diagnostics. Interrogation of chromatin modifications could serve as a tool for diagnosis and monitoring the effectiveness of treatment. In nonmammalian organisms, such as drosophila, chromatin profiling would provide a means of identifying transcriptional target genes and cofactor recruitment during development.³

High-resolution imaging methods, facilitated by stretching and immobilization of chromatin, may provide a direct approach to identifying epigenetic modifications throughout the genome. While the majority of current studies investigate large cell populations through chromatin immunoprecipitation protocols (ChIP) coupled with either hybridization arrays or sequencing⁴ or through fluorescence *in situ* hybridization (FISH)-like visualization at the gross levels of chromosomal superstructure,⁵ few studies

ABSTRACT



Individual chromatin molecules contain valuable genetic and epigenetic information. To date, there have not been reliable techniques available for the controlled stretching and manipulation of individual chromatin fragments for high-resolution imaging and analysis of these molecules. We report the controlled stretching of single chromatin fragments extracted from two different cancerous cell types (M091 and HeLa) characterized through fluorescence microscopy and atomic force microscopy (AFM). Our method combines soft lithography with molecular stretching to form ordered arrays of more than 250 000 individual chromatin fragments immobilized into a beads-on-a-string structure on a solid transparent support. Using fluorescence microscopy and AFM, we verified the presence of histone proteins after the stretching and transfer process.

KEYWORDS: mammalian chromatin · single molecule · soft lithography · molecular stretching · fluorescence imaging · AFM

are aimed toward studying chromatin at the fine scale. The concept of stretching nucleic acids for analysis has been largely exploited in the case of bare DNA. Current techniques for DNA stretching comprise end-tethering, in combination with optical or magnetic tweezers,^{6,7} stretching on polydimethylsiloxane (PDMS) stamps,^{8–13} adsorption onto a modified surface under flow,^{14,15} shear flow,^{16–19} and nanoconfinement.^{20,21} While these techniques should be transposable to chromatin, we note that chromatin stretching is less studied, except for end-tethered stretching^{22–27} and a few recent nanoconfinement studies.^{28,29} The most popular stretching technique for FISH-like genetic

* Address correspondence to hgc1@cornell.edu.

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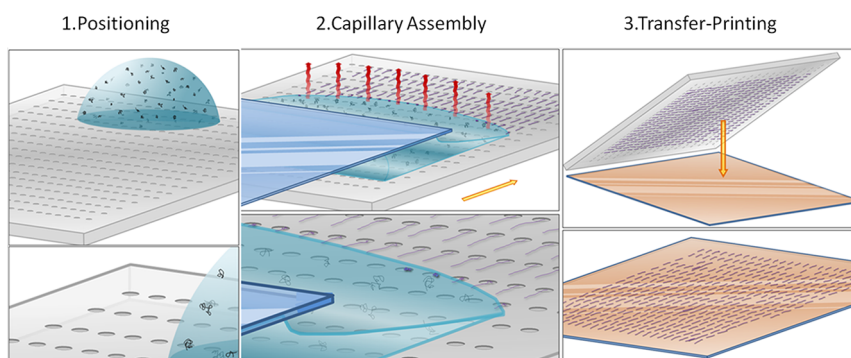


Figure 1. Schematic representation of the experimental methodology to generate stretched and oriented chromatin arrays on a solid support. (1,2) Assembly and stretching process by capillarity. (1) Microstructured PDMS stamp is placed on a translation stage controlled in speed. A droplet of extracted chromatin in solution is deposited on the stamp. (2) Liquid meniscus of the solution containing the extracted chromatin is dragged over the microstructured PDMS stamp at controlled speed. The evaporation phenomenon is represented in red. The molecules are physically trapped and stretched as the meniscus is displaced across the substrate. (3) Transfer printing of the obtained chromatin array on an APTES-coated coverslip by contacting the PDMS stamp with the APTES-coated surface for 2 min and then peeling it away.

or epigenetic analysis relies on cell shearing after fixation, the latter leading to poor molecule to molecule repeatability.^{5,30} As an example, chromatin fibers from drosophila and human cells have been spread by direct lysis onto charged microscope slides to show the organization of covalently modified histones in silent chromatin sequences,³¹ DNA replication timing in centromeric regions,^{32–34} and to study distribution of GINS complex.³⁵ Other studies involve stretching chromatin fibers to establish a mapping between spatial location and genomic location^{5,14} or for morphological studies using transmission electron microscopy and atomic force microscopy.³⁶ In recent years, progress has been made in employing new technologies with the potential to bridge the gap between static structural features and dynamic physiological processes in the study of biomolecules, while increasing spatial resolution.^{37–39} Yet, to our knowledge, no method has been demonstrated that allows the controllable stretching and isolation of canonical and native chromatin molecules for high-throughput analyses while being compatible with high-resolution imaging techniques.

We present a method for the stretching of chromatin molecules into ordered arrays on solid platforms compatible with scanning probe and optical imaging techniques. The controlled stretching of chromatin enables high-throughput location-resolved optical investigation of DNA and nucleoproteic material with a resolution set by signal averaging and the imaging technique employed. We believe that this level of single-molecule analysis is necessary to identify spatial relationships among chromatin features which provide insights into regulatory processes controlling normal chromatin states and how they are disrupted in disease, for studying epigenetic variability in mammalian heterogeneous samples and also for fundamental understanding of biological processes in nonmammalian organisms such as drosophila.

RESULTS AND DISCUSSION

The experimental procedure is depicted in Figure 1. Our methodology is a combination of soft lithography and molecular stretching that allows controlling the number, positioning, and stretching of the chromatin molecules by capillarity during the assembly process. The resulting array is then transferred onto the analysis support by simple contact between the elastomeric stamp and the support.

We first focused our study on chromatin extracted from M091 cells. As a control experiment and to allow visualization of chromatin, DNA was stained with YOYO-1. Figure 2 shows the results obtained from directed capillary assembly of YOYO-1-stained M091 chromatin on a structured PDMS stamp and its subsequent transfer printing onto an APTES-coated coverslip through electrostatic interaction. The fluorescence images show that we obtain, after transfer, ordered and high density arrays of stretched and isolated fragments following a Poisson distribution and over large areas (Supporting Information S1). The size distribution of the chromatin fragments ranges from about 5 to 70 kbp, which corresponds to the expected lengths from the electrophoresis gel (see Supporting Information S2).

The morphology of the stretched fragments appears to be different from the morphology of naked DNA.^{8,40} In some cases, we observe the presence of bright blobs along the molecules. We suggest these inhomogeneities could be caused by wrapping of stained DNA inducing a local increase of fluorescence, uneven staining of DNA, a nod of nucleosomes, bent chromatin, or the presence of more than one fragment of chromatin. We used AFM imaging to confirm the chromatin structure, and Figure 3 shows the obtained AFM images of the sample shown in Figure 2. We observe that the chromatin molecules we see in fluorescence (Figure 2) are decorated with complexes regularly spaced and evenly distributed along the entire fragments' length. The cross-section measurements indicate the average height of each complex to be

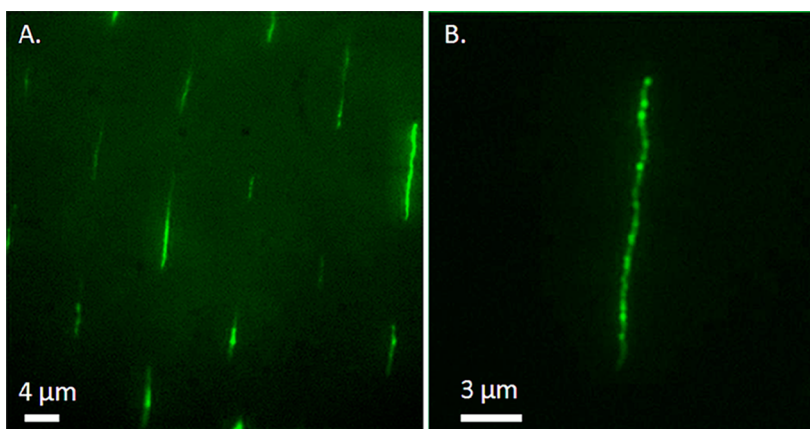


Figure 2. Fluorescence images of an array of stretched and oriented M091 chromatin molecules transferred onto an APTES-coated coverslip (excitation at 475 nm). The molecules are YOYO-1 stained. (B) A 1.5× zoom of image (A).

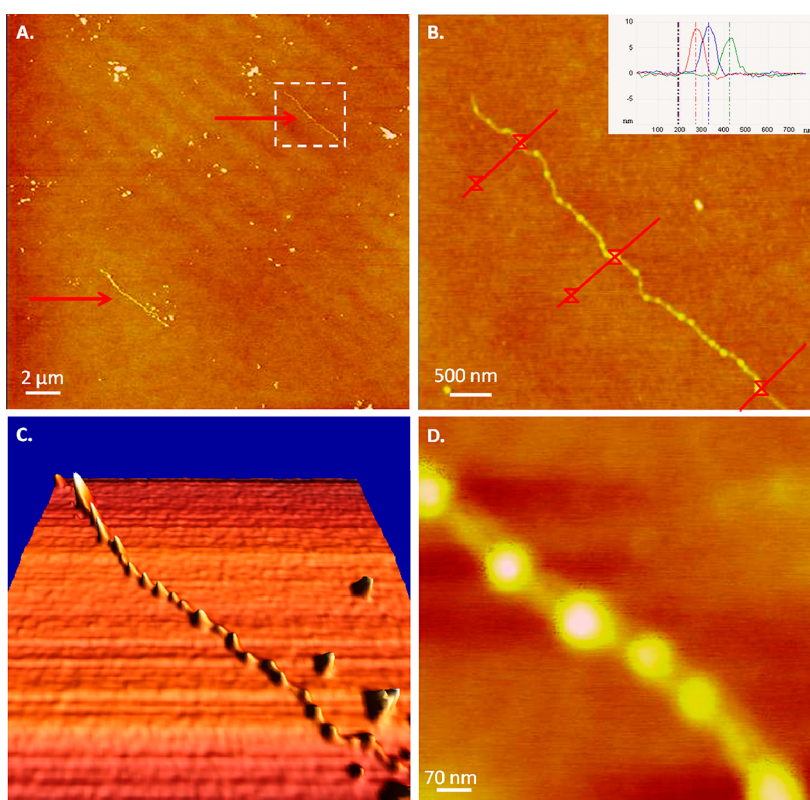


Figure 3. AFM imaging of stretched and isolated chromatin molecule arrays. (A) Large scan view where we notice the positioning of two chromatin molecules as indicated by the red arrows. (B) Enlargement of the area outlined in (A). The inset corresponds to the cross-section measurements performed across the chromatin segment. (C) Three-dimensional representation of the chromatin molecule shown in (B). (D) High-resolution image of the chromatin molecule showing nucleosome distribution and positioning along one section of the stretched chromatin molecule.

10.4 ± 1.8 nm with 136.9 ± 45.0 nm interdistance between adjacent ones. Between complexes, the height of linker DNA was measured at 1.76 ± 0.38 nm. These values are consistent with the theoretical size of nucleosomes in mammalian chromatin which measure 10 nm in diameter on average⁴¹ and are generally well-organized despite cell heterogeneity. This suggests we are in the presence of individual nucleosomes across the fragments, further implying that the technique allows the stretching

of the chromatin fragments to a beads-on-a-string configuration. Note no cross-linking of chromatin molecules was required to fix the histones to the DNA in our process. As such, the forces exerted on the chromatin molecules during capillary assembly are difficult to compare with previous simulations that have been reported. On the one hand, we find simulations of molecular combing on bare DNA molecules onto flat silanized or chemically functionalized surfaces⁴² but no simulations involving chromatin

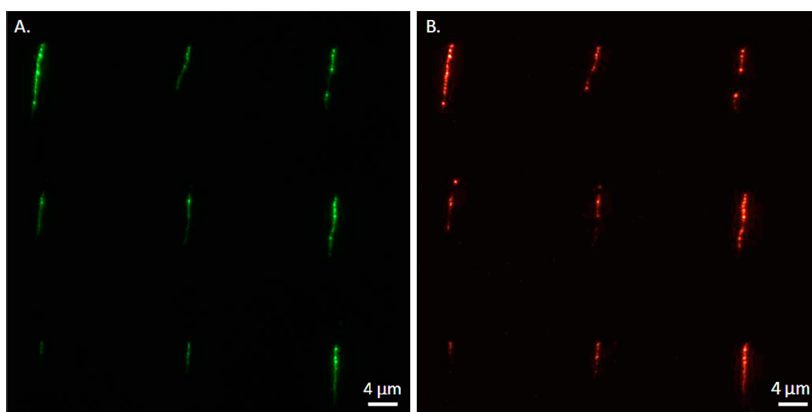


Figure 4. Fluorescence micrograph of M091 chromatin fragments labeled with Alexa647-labeled histone H3 probes. DNA is stained with YOYO-1. (A). Fluorescence micrograph taken at 475 nm excitation. (B). Fluorescence micrograph from the same area taken at 620 nm. Histones H3 (shown in false red, panel B) are colocalized with DNA (shown in false green, panel A).

molecules to our knowledge. On the other hand, we find simulations on the pulling of chromatin molecules using optical traps or magnetic tweezers, where one end of the molecule is chemically modified so it attaches to the support and the other end is attached to a dielectric or paramagnetic bead through a biotin/streptavidin linkage and subjected to a traction or torsion force.^{25,27} In both approaches, DNA is chemically attached from one end while leaving the other end loose and receptive to the stretching force. However, neither of these techniques directly applies to our situation where the molecule is not tethered at one end and neither involves the use of topographical structures of any sort. In our case, we believe there is no chemical attachment but rather that the force elastically stretches the molecule in the immediate vicinity of the meniscus, temporarily “gluing” it to the surface as the meniscus recedes. As such, the pattern geometry plays here an important role as the direction and magnitude of the capillary forces induced by the contact line pinning depend strongly on it.⁴³ From literature, we know that on a hydrophobic substrate, the force exerted by flow combing is on the order of ~ 160 pN,⁴² and that overstretching and denaturation of naked DNA is observed beyond 150–300 pN depending on sequence.⁴⁴ However, are the elastic properties of chromatin comparable to the elastic properties of bare DNA? Optical trapping is one of the few techniques that have been able to provide insights into nucleosome dynamics. We know that trapping forces typically range between 0.1 and 100 pN, making this technique suited for investigation in the range of 0.01 and 10 pN.⁴⁴ On the basis of the work of Wang *et al.*,²⁵ we also know that the pulling force required to strip off nucleosomes from chromatin is on the order of tens of picoNewtons. Our method differs from molecular combing, and some key variants make the overall stretching force difficult to evaluate. In our experiment, the forces involved are an overall combination of various contributions (van der Waals forces, hydrodynamic forces, substrate displacement, capillary force, convective flow, hydrophobic interactions). What we

observe, however, is that the pinning of the molecules provides them with same alignment and orientation, that the resulting lengths are comparable to the lengths expected from the electrophoresis gels, and finally, that the nucleosomes are preserved during the process with interdistances kept constant and consistent with theory. These observations suggest that the forces exerted upon the fragments during the capillary assembly process are low enough to prevent the stripping of histones but at the same time high enough to allow the stretching of the fragments into the beads-on-a-string conformation necessary for high-resolution analysis. Thus, we can infer that with our substrate design and experimental parameters with a stage displacement speed of $20 \mu\text{m/s}$, the overall force is somewhat comprised between a few picoNewtons to 100 pN. This ability to isolate individual chromatin fragments from an initial compacted higher-order form in solution refined into a beads-on-a-string conformation without the need for cross-linking is a characteristic of our technique which is essential for subsequent analysis. It gives access and insights into the entire library of information contained within individual chromatin fragments while preserving their native structure.

To confirm the preservation of native chromatin fragments, we interrogated the extracted chromatin fragments from M091 cells with histone H3 labels. For histone labeling, we used immunolabeling through specific recognition between a histone primary antibody and its corresponding fluorescently labeled secondary antibody. To validate the specificity of our labels, we first conducted several control experiments where only fluorescent secondary antibodies were incubated against chromatin, without any primary histone antibody intermediate (Supporting Information S3). The results showed a very low rate of non-specific binding compared to the specific binding of histone antibodies of interest. In fact, since the process takes place on a hydrophobic substrate (PDMS) and since the assembly only acts on the long-chain polymer

strands, this method inherently washes away unbound or free dyes and other contaminants. Consequently, no prior purification or washing steps are required. The specificity of the antibodies being validated, we repeated the experiments but this time using the specific probes of interest. The results from our chromatin immunolabeling are shown in Figure 4. The density of molecules in the arrays allows for histone distribution to be statistically compared across each sample and between different samples. We observe in Figure 4b that, as expected from our AFM imaging, Alexa647-labeled histone H3 antibodies (shown in false red color) are colocalized with the molecules and densely distributed across the entire length of the molecules. We notice that some fluorescent spots are brighter than others. These inhomogeneities that were already observed in Figure 2 could also be caused here by the presence of antibody aggregates at some locations across the stretched molecules. This experimental protocol was repeated with chromatin extracted from HeLa cells. As shown by the electrophoresis gel (Supporting Information S2), the chromatin extraction from HeLa cells required lower micrococcal nuclease concentration and shorter digestion times to provide us with equivalent fragment sizes. Conversely, the experimental parameters used for stretching and generation of the HeLa chromatin arrays were kept identical, and similar results were obtained (see Supporting Information S4–S6).

Considering the number of chromatin fragments per chip and the average length of the fragments, a simple calculation allows us to roughly estimate that we could have about a human genome amount of DNA per chip. This suggests that our technique would allow the imaging of an entire genome in a single experiment through a fast optical readout.

For data to be biologically relevant, the precision has to be at least one gene, or about 10 000 base pairs (bp). This implies that, given the diffraction limit of optical microscopy of about 300 nm, chromatin has to be stretched to at least 30 bp/nm. If we analyze the panel of fluorescent images in Figure 4, the signals, even from the two closest probes of a 1 kbp separation distance, are clearly discernible (Figure 4; the mean space measured between the two signals is 320 nm), from which we can conclude the resolution of our technique to be at least within 320 nm in fluorescence. However, this resolution can be easily increased by coupling fluorescence analysis with a nanometer resolution technique such as AFM. Furthermore, as this methodology can be applied to various hydrophilic

supports, this resolution could be further improved by using high-resolution fluorescence microscopy techniques such as total internal reflection fluorescence (TIRF), photoactivated localization microscopy (PALM), or stochastic optical reconstruction microscopy (STORM).⁴⁵ It is not unreasonable to extrapolate that the fundamental principles outlined in our technique may permit molecular mapping and analysis with sufficient spatial resolution to identify multiple epigenetic marks on a single nucleosome or to distinguish marks on adjacent nucleosomes in native chromatin. Additionally, we could also envision coupling this chromatin array chip with optical tweezers for high-throughput native chromatin assays.

CONCLUSIONS

In summary, we have demonstrated a methodology to stretch and immobilize single native chromatin fragments derived from a mammalian genome, into a beads-on-a-string conformation. Our technique allows controlling the stretching factor of chromatin molecules to prevent stripping of histones from the molecules. We proved the applicability of this method by employing it in the case of two different cancerous cell types (M091 and HeLa). Thus, this procedure could be transposed, by extension, to other mammalian or nonmammalian cells of interest (embryonic stem cells, drosophila, for example). This ability to controllably pattern large numbers of chromatin molecules over square millimeter areas offers the opportunity for parallelized and high-throughput screening with high-resolution capabilities. Therefore, chromatin mapping represents a powerful technique with the potential to serve as the basis of a FISH-like technique providing single-molecule maps of multiple epigenetic features and their relation to a specific gene for single-cell diagnostics. This technique could be extended to the use of microfluidics for dealing with material extracted from a single cell.¹⁹

In addition to research studies, this affordable and fast technique may prove to be especially well-tailored for clinical settings as the procedure is straightforward and does not require complex fabrication or preparation protocols. In the imminent future, the need will arise to investigate the relationships between genetic and epigenetic marks not only within localized regions on the genome but also among different cell types. This may grant new insight into the relation between chromatin structure and mechanisms of gene regulation during cellular differentiation and development as a means of bringing hope to tomorrow's medical treatment options and diagnostics.

METHODS

Preparation of Biological Materials. *Chromatin Extraction.* Acute myelogenous leukemia-derived cells (M091)⁴⁶ were cultured as

a suspension in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum at a density of 10^5 cells/mL. HeLa cells were cultured as monolayers in DMEM supplemented

with 5% fetal calf serum. HeLa cells were plated at a density of 10^5 cells per plate. When the cells reached 80–90% confluence, native chromatin fragments were extracted as described.^{29,47} Micrococcal nuclease (NEB M02475) digestion times were set to 30 min (2000 gel units) and 5 min (200 gel units) for M091 and HeLa chromatin, respectively. Extracted chromatin was resuspended in a final buffer of 10 mM EDTA and 0.5 M NaCl. The resulting fragment size distribution of extracted chromatin was evaluated through an electrophoresis gel (see Supporting Information, Figure S2).

Extracted chromatin was stained with YOYO-1 (Invitrogen, 100 μ M) at a 1:5 dye/base ratio for control experiments.

Antibody Labels. Histone H3 monoclonal antibodies (39763) were purchased from Active Motif. Alexa647 goat anti-mouse secondary antibodies were purchased from Invitrogen. Both primary and secondary antibodies were used at 1:200 dilution.

Histone Labeling. Two micrograms of M091 or HeLa chromatin was separately incubated against histone primary antibodies for 1 h at room temperature. Secondary antibodies were then added to the solution in equal proportion as primary antibodies and incubated for an additional 30 min at room temperature. The entire solution was then counter-stained with YOYO-1 intercalating dye (Invitrogen) for a minimum of 30 min in the dark at room temperature. Finally, 15 μ L of $1 \times$ PBS with 0.3% Triton X-100 v/v was added to adjust the contact angle of the solution with respect to the PDMS surface (measured contact angle of 45° in water). The total volume was used for each directed assembly experiment.

Operational Procedure. To direct the capillary assembly of digested chromatin, we used PDMS stamps with topographical cavities obtained from the replication of a positive silicon master. Conventional photolithographic techniques (proximity UV lithography) and reactive ion etching (RIE) were used to fabricate a silicon master with the negative of the final desired micropatterns. The design consists of arrays of 5 and 8 μ m in diameter, 5 μ m high protruding features, with different periodicities (15 and 20 μ m). The PDMS prepolymer solution containing a mixture of 10:1 mass ratio of PDMS oligomers and reticular agent from Sylgard 184 Kit (Dow Corning, Wilmington, DE) was then molded against the silicon master. An anti-adhesive treatment of the master was previously carried out using silanization in liquid phase with octadecyltrichlorosilane (OTS) in order to enable easy demolding of the polymer replica after thermal curing. PDMS was cured at a temperature of 80°C for 2 h. Once demolded, the PDMS substrate was carefully cut into $1.8\text{ cm} \times 1.4\text{ cm}$ stamps. Next, to conduct the directed capillary assembly, we use a dedicated setup. The resulting PDMS stamp where we want the chromatin fragments to be assembled is placed on a motorized translation stage below a fixed glass spreader at a distance of about 1 mm. The experiment is conducted at ambient temperature. The chromatin solution is injected between the glass and the substrate, and the liquid contact line is then moved over the substrate at a finely adjusted and constant velocity of 20 $\mu\text{m/s}$ for the chromatin fragments to be carefully trapped and stretched. The main parameters governing this process are the molecular concentration, the velocity of the translation stage, the contact angle at the liquid/substrate/vapor interface, and the evaporation rate of the solvent. In this physical process, the microwell acts as a wetting defect that temporarily pins the moving contact line. During this pinning time, the convective flux of molecules in solution nourishes the interface, the contact angle of the liquid front line locally decreases, and the assembly takes place selectively by capillarity at each well. Under stretching of the meniscus due to the motion of the stage, some elastic energy is stored. When this energy exceeds the pinning energy, the front line abruptly disrupts, simultaneously stretching the trapped molecules and fixing them in that position. The assembly is performed throughout the entire surface of the PDMS stamp of an approximate 1 cm^2 area (see Supporting Information S1 and S4). To transfer the formed chromatin arrays onto the analysis support, the PDMS stamp with assembled chromatin fragments is brought into contact with a (3-aminopropyl)triethoxysilane (APTES)-coated coverslip for 2 min and then peeled away (Figure 1). The chromatin array transfers were characterized

under an inverted epifluorescence microscope (100 \times oil immersion objective) from Olympus coupled to a 512×512 CCD camera (Photometrics). The samples were imaged under illumination at 475 nm ($\text{em} = 535\text{ nm}$) and 620 nm ($\text{em} = 700\text{ nm}$) accordingly with no detectable cross-excitation at any of the emission wavelengths. Images were combined using ImageJ software.

AFM Imaging. AFM images of chromatin arrays on APTES-coated glass slides were obtained on a NanoScope IIIa (Digital Instruments) in tapping mode operated in air.

Conflict of Interest: The authors declare no competing financial interest.

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Supporting Information Available: Electrophoresis gels and control experiments were included as supporting information, along with videos taken from samples, and fluorescence and AFM images obtained with chromatin extracted from HeLa cells. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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