Super-Resolution Genome Mapping in Silicon Nanochannels

Jonathan Jeffet†, Asaf Kobo†, Tianxiang Su⊥, Assaf Grunwald†, Ori Green†, Adam N. Nilsson§, Eli Eisenberg†, Tobias Ambjörnsson§, Fredrik Westerlund∥, Elmar Weinhold∇, Doron Shabat†, Prashant K. Purohit‡, and Yuval Ebenstein*†

†Raymond and Beverly Sackler Faculty of Exact Sciences, Tel Aviv University, Tel Aviv 6997801, Israel
‡Department of Mechanical Engineering and Applied Mechanics, University of Pennsylvania, Philadelphia, Pennsylvania 19104, United States
⊥School of Engineering and Applied Sciences, Harvard University, Cambridge, Massachusetts 02138, United States
§Department of Astronomy and Theoretical Physics, Lund University, SE-221 00 Lund, Sweden
∥Department of Biology and Biological Engineering, Chalmers University of Technology, SE-412 96 Gothenburg, Sweden
∇Institute of Organic Chemistry, RWTH Aachen University, Aachen D-52056, Germany

ABSTRACT: Optical genome mapping in nanochannels is a powerful genetic analysis method, complementary to deoxyribonucleic acid (DNA) sequencing. The method is based on detecting a pattern of fluorescent labels attached along individual DNA molecules. When such molecules are extended in nanochannels, the labels create a fluorescent genetic barcode that is used for mapping the DNA molecule to its genomic locus and identifying large-scale variation from the genome reference. Mapping resolution is currently limited by two main factors: the optical diffraction limit and the thermal fluctuations of DNA molecules suspended in the nanochannels. Here, we utilize single-molecule tracking and super-resolution localization in order to improve the mapping accuracy and resolving power of this genome mapping technique and achieve a 15-fold increase in resolving power compared to currently practiced methods. We took advantage of a naturally occurring genetic repeat array and labeled each repeat with custom-designed Trolox conjugated fluorophores for enhanced photostability. This model system allowed us to acquire extremely long image sequences of the equally spaced fluorescent markers along DNA molecules, enabling detailed characterization of nanoconfined DNA dynamics and quantitative comparison to the Odijk theory for confined polymer chains. We present a simple method to overcome the thermal fluctuations in the nanochannels and exploit single-step photobleaching to resolve subdiffraction spaced fluorescent markers along fluctuating DNA molecules with ∼100 bp resolution. In addition, we show how time-averaging over just ∼50 frames of 40 ms enhances mapping accuracy, improves mapping P-value cores by 3 orders of magnitude compared to nonaveraged alignment, and provides a significant advantage for analyzing structural variations between DNA molecules with similar sequence composition.

KEYWORDS: nanochannels, super-resolution, single-molecule, optical genome mapping, DNA labeling, confined polymers

With recent advancements in nanofabrication and single-molecule microscopy, optical genome mapping has reemerged as a valuable complement to deoxyribonucleic acid (DNA) sequencing. The method is based on labeling long DNA fragments with fluorescent molecules indicating specific sequence motifs to create a specific pattern along the DNA that serves as a barcode for genetic identification. (Figure 1A). The DNA is then extended in nanofluidic channels and visualized by fluorescence microscopy, revealing the DNA contour decorated with a pattern of fluorescent spots.
The relative position of the spots is aligned with an *in silico* reference map of expected label positions that has been constructed based on the known genome sequence.

**Figure 1B.** The relative position of the spots is aligned with an *in silico* reference map of expected label positions that has been constructed based on the known genome sequence.

**Figure 1.** DNA model molecule confined in nanochannels. (A) Schematic illustration of the sequence specific, two-step labeling process. First an azide group is attached to the adenine base of a TCGA sequence by a DNA methyltransferase enzyme (M.TaqI). Next a Cy5 fluorophore, conjugated to a Trolox triplet-state quencher (see Supporting Information for specific synthesize protocol), is connected to the azide group by a DBCO alkyne group via a copper-free click reaction. (B, left) Scanning electron microscope (SEM) image of a silicon nanochannel array; the bar represents 1 μm. (B, right) Cropped images of BAC molecules stretched and imaged inside the nanochannels (YOYO-1 in red, fluorescent Cy5 labels in green). (C) BAC molecule overstretched on an activated coverslip (stretching factor of ∼2 compared to the nanochannels), which enables resolving the two fluorophores composing each fluorescent spot observed in the nanochannels.

Diffraction limited optical imaging restricts the ability to resolve two adjacent fluorophores that are separated by less than about half the detected wavelength. Depending on the exact degree of extension in the channels, this distance translates to approximately ∼1000 bp. Consequently, the current state of the art Irys platform from BioNano Genomics Inc. resolves labels separated by 1500 bp and above. In a typical experiment, a snapshot of the DNA molecule is taken in order to reveal the pattern of fluorescent spots along the DNA contour. Seemingly, the linear configuration of the DNA molecules provides the perfect template for super-resolution localization and mapping of DNA, and indeed fluorescent labels may be localized with nanometer precision in the nanochannels. However, since the DNA molecules remain suspended in solution inside the channels, they undergo thermal fluctuations and are captured at a nonequilibrium position that contributes to the theoretical uncertainty in genetic label position. Accordingly, the physics of DNA in nanochannels influences two aspects of genome mapping: information density and mapping accuracy. The maximum achievable information density is set by the diffraction limit and by fast thermal fluctuations that occur within the duration of a single imaging exposure. These define the effective fluorescent spot size and, therefore, the maximum possible number of resolved labels along a DNA molecule. The mapping accuracy, on the other hand, defined by the degree of correlation between the resolved...
optical map and the expected genetic label positions, is limited by the long time scale deviation of labels from their equilibrium positions due to DNA fluctuations.

Here, we address the question of super-resolution mapping from a practical perspective and define the various limits of resolution and mapping accuracy in such nanochannel mapping systems. We find that the thermal fluctuations of the DNA molecules impose the critical limit on achievable accuracy and restrict it to the order of 1–2 kilobase pairs (kbp) with single frame analysis. On the other hand, when taking multiple frames of the same molecule, mapping accuracy is increased, and super-resolution imaging becomes possible. By time averaging over multiple frames, we were able to reconstruct the relative equilibrium positions of genomic labels and to provide information about the underlying sequence with accuracy on the order of 300 bp. This enhanced accuracy is critical when trying to resolve structural variations between genomic regions with similar sequence composition.

In order to reliably characterize the resolution enhancement offered by super-resolution localization, we take advantage of a naturally occurring macro satellite array residing in human chromosome 4.16 This region is composed of multiple repeats of a 3.3 kbp sequence that was cloned into a bacterial artificial chromosome (BAC) vector, allowing the generation of multiple copies of this genomic region for optical analysis. The repeatcontaining BAC displays equally spaced fluorescent markers along the DNA molecules with precisely known genomic spacing. Furthermore, each fluorescent spot is composed of two fluorophores spaced 676 bp apart that serve to test our ability to resolve small (subdiffraction) genetic distances between labels (Figure 1 B, C).

In order to confirm the predicted fluorescent pattern, we first stretched our DNA model system on chemically modified microscope cover slides that allow overstretching of the deposited DNA molecules.20 Under these conditions, the periodic fluorescent pattern is directly visualized, indicating a stretching factor of ~1.9 compared to the contour length of B-form DNA, approximately two times the typical extension for DNA in the nanochannels used (Figure 1 B, C). We used silicon nanochannel array chips commercialized by BioNano Genomics Inc. These channels have a ~45 nm square cross section and are routinely used for next generation genome mapping.11,21,22 A solution of labeled DNA molecules was loaded into the chip inlet reservoir and electro-kinetically driven into the channels by applying a voltage across the input and output reservoirs. Labeled molecules, confined and stretched in the channels, were visualized on an inverted fluorescence microscope, appearing as an equispaced string of fluorescent spots. In order to analyze the thermal fluctuations of the DNA molecules, we recorded time lapse movies of the spots at 40 ms intervals and localized the positions of the spots in each frame by 2D Gaussian fitting using the “Localizer” software package.23 We then analyzed the individual tracks recorded for each fluorescent spot in order to assess the extent of the fluctuations and to calculate the time-averaged equilibrium distances between spots. Initial imaging experiments resulted in rapid photobleaching of the Cy5 molecules used for labeling. To obtain sufficient statistics for analysis, much longer trajectories are required. To this end, Trolox was covalently attached to the Cy5 molecules to serve as a triplet quencher stabilizing agent.18,19,24 In conjunction with a protocatechucic acid/protocatechuate 3,4-dioxygenase (PCA/ PCD) oxygen scavenging system,25 we were able to record molecular fluctuations over extended periods, enabling full characterization of the dynamics of DNA molecules in the channels. A kymograph of such a time lapse is presented in Figure 2C.

RESULTS AND DISCUSSION

The Effect of Thermal Fluctuations on Mapping Accuracy. Confinement of DNA molecules in nanochannels has been modeled theoretically by T. Odijk for channel widths smaller than the DNA persistence length (~50 nm with the ionic strength in this experiment).5,14,26–28 The theoretical analysis predicts thermal fluctuations of the DNA chain around its equilibrium configuration, which leads to label localization error (fluctuations faster than the duration of a single frame) and mapping inaccuracies (deviation from the label equilibrium positions). By analyzing the dynamic label locations
extracted from the BAC molecules, we were able to estimate the contribution of fluctuations to mapping inaccuracies common to single-snapshot mapping. We calculated all point to point distances between labels in the repetitive array and found that the maximal displacement from the 3.3 kbp equilibrium distance in our repetitive array was on average ±0.7 kbp (with a fraction of fluctuations reaching up to ±1.2 kbp from equilibrium). Considering that the fluctuations become more significant with increasing label separation distances,\(^\text{8,15,28–30}\) these values correspond well to the estimated errors for optical mapping accepted in the field today (≈1.5 kbp for a 10 kbp distance\(^\text{10,11}\)). The presented method of calculation gives an overall estimation of the inaccuracies in label mapping positions induced both by the thermal fluctuations and by the superresolution localization error. Since the localization error of a single label in this experiment was calculated to be on the order of ~100 bp, it is obvious that the main deviation from equilibrium label positions in optical mapping experiments arises from long time scale thermal fluctuations.

Theoretical Framework for Nanoconfined DNA Molecules. Odijk theory provides a theoretical framework for confined polymers. In order to check whether the theory comprehensively describes our experimental observations, we calculated the point to point distance distribution between all label pairs in the repetitive segment of the BAC molecules. The theoretical point to point distance distribution (eq 1 in the Supporting Information), derived from Odijk’s theory by Su et al.,\(^\text{28}\) is in good agreement with our data (see Figure 3A). From this distance distribution, we could accurately estimate the mean distance in the image plane between repetitive elements (1101 ± 12 nm for 3.3 kbp) and, hence, find the effective stretching factor with very high accuracy. The main deviation of our measurements from the theoretical distribution appears in the discrepancies between the standard deviation (STD) of the measured data and the expected value given by the theory (Figure 3A inset and 3B). As seen before in the experiment of Su et al.,\(^\text{28}\) the STD of distances corresponding to shorter contour lengths does not follow the expected root law, but rather follows a broader distribution which could not be accounted for by the fluorophores’ localization error (as shown in the Supporting Information). The conclusion from this result is that, unlike the theoretical prediction of diminishing fluctuations at short label distances, the accuracy improvement is actually limited and the fluctuations remain significant even at a 3.3 kbp separation. This discrepancy can be explained by the fact that the actual channel width has dimensions on the order of the DNA persistence length (D ≈ ξp), while Odijk theory requires much smaller channel dimensions (D << ξp), as has been suggested by Reinhart et al.\(^\text{13}\) and shown in simulations by Muralidhar et al.\(^\text{27}\) Furthermore, a comparison between different molecules from the same experiment revealed a distribution of physical DNA repeat lengths. One plausible explanation for this variation is nonhomogeneous staining of the DNA backbone by the intercalating dye YOYO-1, which is known...
to effect the DNA contour length.\textsuperscript{31–33} By applying Odijk’s theory, we were able to accurately calculate the different YOYO-1 labeling densities for different molecules from the same batch (see Figure S2). This suggests that even when YOYO-1 staining is homogeneous along individual molecules, the different labeling densities on different molecules could result in a nonconstant stretching factor, as has been shown by Nyberg et al.\textsuperscript{33}

**Figure 3.** Comparison to Odijk theory. (A) Point to point distance distribution according to Odijk’s theory (black) plotted over the distribution of distances between all labels calculated in all frames. The first five peaks are presented in the inset to show the discrepancy between the estimated and the observed STDs. (B) Mean distances between close neighbor labels (data points) and their STD (error bars), compared to the theoretical prediction of mean distance and STD by Odijk’s theory. The gray rectangles show the ±3σ theoretical regions around the mean distance value, which follow a square root law with distance (n signifies the integer number of 3.3 kbp distance between labels).

**Accuracy Enhancement by Time-Averaging.** The average location of fluorescent labels can be calculated in two different ways: either by calculating the average spot locations using the super-resolved coordinates from each frame\textsuperscript{34} or by calculating the distances between consecutive spots in each frame and accumulating the mean distances to create a locations map. Although this might appear to be merely a semantic difference, the latter option filters out the collective modes of vibration of the entire DNA molecule, which reduces the fluctuations’ STD by a factor of 2. This is clearly demonstrated in Figure 4, where the STD of the distance averaging method is significantly reduced, as compared to the mean location analysis. Moreover, the distance averaging contributes significantly to the mapping accuracy. To show this, alignment to the theoretical sequence was conducted via two different analysis methods: cross-correlation analysis, using custom software based on the work of Nilsson et al.\textsuperscript{5,35,36} and genome alignment, using the commercial IrysView software package from BioNano Genomics\textsuperscript{9} (see Supporting Information for confidence scoring method). The results show a significant improvement in the alignment score when using the averaged distance analysis, compared to using the averaged location analysis or single-frame snapshots (Figure 5B). As seen in Figure 5B, a larger amount of detected labels leads to an increase in the alignment confidence, emphasizing the advantage of multiframe analysis for handling blinking events, as explained in the Supporting Information. In addition, averaging over only ~50 frames of 40 ms was sufficient in order to achieve a significant improvement in the accuracy and alignment scores, drastically reducing the time consumption of this method compared to the 1000 frames collected initially (Figure 5C). We note that alignment confidence increases rapidly with the number of frames and so the trade-off between mapping quality and acquisition time may be tuned according to the demands of the specific experiment. With this distance averaging method, the main requirement is that the distance between consecutive spots would be <~10 kbp, because of the decrease of fluctuation correlation with distance (see Figure S12).
Figure 4. Location versus distance averaging. (A) Fitted locations kymograph. In each frame, all labels were localized by 2D Gaussian fits with $\sim 30$ nm accuracy. (B) Distances kymograph. In each frame the distances between adjacent labels were calculated and then accumulated to give effective locations. The global fluctuations of the molecules are greatly reduced in comparison to (A). (C) Comparison of the STD in position for each label between the two methods. (D) Displacement from equilibrium; comparison of location versus distance. For each spot (or distance between two adjacent spots), the locations (distances) in each frame were subtracted from the mean spot location (mean distance between spots) and plotted as a histogram with the sum of counts normalized to unity. It is clear that the location distribution has a higher STD compared to the distance distribution.

Figure 5. Mapping accuracy enhancement via time-averaging. Mapping accuracy was assessed using the IrysView program. We compared between the averaged label locations (green bars), calculated according to the two analysis methods described in the text, and label locations in single-frame snapshots (purple bars). Label locations from the different calculations were aligned to the theoretical reference map, and the alignment confidence score was calculated for the different scenarios. (A) Visual representation of the molecules’ alignment to the theoretical sequence generated by IrysView. Molecules are represented as yellow lines with labels marked as blue bars. The theoretical sequence is presented on top with the expected TCGA labeling sites marked as blue lines along the molecule. (B) IrysView alignment score comparison between the averaged label locations (green bars) and single-frame label location alignment (purple bars). The blue and red backgrounds represent the amount of labels that were aligned: blue for 21 labels and red for 19 labels due to bleaching and blinking effects. (C) Alignment score as a function of the amount of frames used for time-averaging. It is evident that a significant increase in confidence score is achieved with as little as $\sim 50$ frames, which, in principle, would increase acquisition time up to $\sim 10$ times the acquisition time of common genome mapping in nanochannels ($50$ frames of $40$ ms compared to the standard $200$ ms single snapshot exposure).

Thermal Fluctuations and Their Effect on Super-Resolution. The fluctuations in label location (average STD of $\sim 410$ bp, or $\sim 136$ nm) prohibit resolution of two subdiffraction-separated labels with conventional super-resolution and localization methods$^6,12,37,38$ (see Figure S11). In each frame, we achieve high-resolution label localization ($\sim 30$ nm), but the fluctuations dictate the actual resolving power because of the smearing effect due to deviation from the equilibrium position. Even when employing super-resolution techniques that exploit the blinking or photobleaching behavior of the fluorescent dyes, the rapid fluctuations prohibit the assignment of a meaningful genetic location, because it most likely captures the label at a nonequilibrium position (see Figure S11). However, as
discussed above, calculation of the distances between resolved labels makes it possible to significantly reduce these fluctuations by elimination of the global DNA molecule displacement. Thus, using a fluorescent mark with constant intensity as a reference point on the DNA to account for local chain fluctuations, one can use the blinking or bleaching behavior of a second target mark to resolve the inner structure of fluorescent spots originating from multiple fluorophores, even if they are separated by distances shorter than the diffraction limit (see illustration in Figure 6A). In Figure 6 the bleaching event generates a step response in the distance between the target mark and the reference point and thus reports on the actual subdiffraction distance between the fluorophores composing the target spot. When using the closest label (3.3 kbp) as a reference point, the calculated distance between the fluorophores from this bleaching event is 670 ± 130 bp (224 ± 44 nm), in good agreement with the theoretical distance between the fluorophores (676 bp). Using this approach we were able, first, to reliably map the 3.3 kbp repeat units by time-averaging over the spot to spot distances and then, by considering bleaching events, to resolve the subdiffraction-spaced labels composing each repeat spot. For additional discussion on the requirements for the success of this super-resolution method, we refer the reader to the Supporting Information.

Figure 6. Super-resolution of subdiffraction spaced genetic labels. (A) Schematic illustration of the method. Each fluorescent spot in our model DNA molecule is composed of two fluorophores separated by a subdiffraction distance. The distance between two spots reflects the distance between the centers of the fluorophore pairs. If one of the outer labels bleaches, then the distance between the target spot and the reference spot would decrease by half the distance between the target fluorophores, thus resolving the subdiffraction structure of the target spot. (B) 3D visualization of the intensity versus time for two fluorescent spots separated by a 3.3 kbp repeat unit (the intensity was calculated as the sum of the intensities of the 2D Gaussian fits in each frame). Each spot is composed of 2 fluorophores, where a bleaching event of one of the fluorophores of the left spot is evident after 15 frames. (C) Intensity time trace of the labels calculated from the 2D Gaussian fits. (D) Distance between the two spots as a function of time. It is clear that the bleaching step alters the mean distance between the two spots. The difference in distance is half the distance between the two fluorophores comprising the inner structure of the target spot.
CONCLUSIONS
In this work, we investigated the advantages of multiframe analysis for DNA mapping in nanochannels. We showed that the correlated fluctuations over small genomic distances in the Odijk regime allow increased mapping accuracy as well as the ability to resolve subdiffraction label structures. We measured the physical accuracy for single snapshot mapping to be 1.4–2 kbp (depending on the distance between labels), with the main error contribution being from long time scale thermal fluctuations. Comparison to the theory reveals an additional broadening mechanism at low genomic distances; this may originate from the deviation of our channels’ width from Odijk’s theory assumptions and/or from variation in DNA stretching factor arising from nonuniformity in YOYO-1 staining between different molecules. We showed that averaging label locations over 50 frames enhances mapping confidence and enables localization of labels with ~300 bp accuracy, as opposed to the 1500 bp limit currently defined by BioNano Genomics. In special cases higher resolution becomes possible by applying super-resolution techniques. We show that by using the single-step photobleaching properties of single fluorophores, we can resolve two labels 676 bp apart and calculated the limit of resolution using this technique to be ~100 bp, indicating a 15-fold increase compared to the current limit of resolution. Together, our findings indicate that by applying simple measures, standard genome mapping accuracy can be increased by a factor of 5, and mapping scores are increased by up to 50%. However, this comes at the cost of a factor of 10 in acquisition time. This approach may prove useful for highlighting small genetic differences between DNA molecules or for difficult-to-assemble genomes. Furthermore, by use of the proposed super-resolution technique, one can zoom in on specific regions in order to define their fine genetic structure. Finally, by the ability to map denser fluorescent barcodes, our approach may serve to increase the information content offered by optical genome maps and extend the range of genetic motifs applicable for genetic mapping.

METHODS

DNA Labeling and Preparation. E. coli cells, containing the CH16-291A23 BAC, were cultured for 12 h in LB containing 12.5 μg/mL chloramphenicol (Sigma-Aldrich, Rehovot, Israel) at 30 °C. The BAC DNA was specifically purified from the cells and the bacterial genomic DNA using the NucleoBondXtra BAC kit (MACHEREYNAGEL Inc. Düren, Germany). The purified DNA was linearized using the restriction enzyme NotI (New England Biolabs Inc. Ipswich MA, U.S.A.). Fluorescent, sequence specific, labeling was generated using the DNA MTase M.TaqI, which catalyzes the transfer of an azide group from the synthetic cofactor AdoHexAzide onto the adenine residue within its recognition sequence (TCGA).5,17 as follows: 1 μg of DNA was reacted with 37.5 ng of M.TaqI and 40 μM of AdoHexAzide in labeling buffer (20 mMTris/POAc, 10 mM Mg(Cl)₂, 50 mM KOAc, 1 mM DTT, pH 7.9) in the presence of 0.01% Triton-X 100 and 0.1 mg/mL BSA in a total reaction volume of 25 μL at 60 °C for 1 h. The labeled DNA was reacted with 40 μg of proteinase K (Sigma-Aldrich, Rehovot, Israel) at 45 °C for 1 h to disassemble protein–DNA aggregates and later was embedded in 1% low melting point agarose plugs and washed three times by TE buffer pH 8. Next, in order to fluorescently tag the DNA, a strain-promoted copper-free click reaction was performed between the azide groups that were attached to the DNA in the previous reaction and 0.5 mM dibenzocyclooctyl (DBCO) + Cy5 + Trolox conjugate (see Figure 2 and Figure S13). Trolox was used to enhance the photostability of the fluorescent Cy5 molecule.24 The labeled DNA was cleaned using ethanol precipitation. Prior to imaging, DNA was stained with 1 μM of YOYO-1 (Invitrogen, Carlsbad, CA, U.S.A.) to obtain visualization of its contour.

Oxygen Scavenging PCD/PCA System for Photostability Enhancement. In order to extend the fluorescence emission time until photobleaching, a PCA/PCD oxygen scavenging system was prepared and used according to an established protocol.25 PCD and PCA were added to the DNA buffer solution to obtain final concentrations of 50 nM and 2.5 mM, respectively, a few minutes before acquiring the data.
**Optical System and Data Acquisition.** Data was acquired using an Olympus IX81 microscope adapted for laser-fluorescence microscopy. A 637 nm CW laser diode (Coherent, OBIS 637LX) was used as an excitation source for the Cy5 fluorescent labels and a 473 nm CW laser (OEM, 200 mW) as an excitation source for the YOYO-1 intercalating fluorescent dye. Excitation light was focused on the back aperture of a 100× oil immersion objective (Olympus, UPlanSApo 100×/N.A 1.4), with an average excitation power density of ~500 W/cm² in the sample plane. Sample fluorescence was collected by the same objective and imaged through a polychroic beamsplitter (Chroma, ZT473/532/637 rpc-xt890) and emission filters (Semrock, 679/41 Brightline, 510/20 Brightline) onto a sCMOS camera (Hamamatsu, ORCA-Flash4.0 V2) with 13 × 13 μm² effective pixel size after pixel binning (130 × 130 nm² in sample plane) and single-fluorophore detection sensitivity. The sample was fixed to an XY stage (Applied Scientific Instrumentation (ASI), Eugene, OR, U.S.A.) to enable scanning of the sample. The equipment and acquisition were controlled by µManager software.\(^{41}\)

**DNA Stretching in Nanochannels.** The stretching of DNA molecules in nanochannels was performed in a commercially available nanochannel array chip (BioNano genomics Inc.).\(^9\) Labeled DNA (suspended in a flow buffer containing: 1.25 mM polyvinylpyrrolidone (Sigma-Aldrich, Rehovot, Israel) and 3% of Tween-20 (Sigma-Aldrich, Rehovot, Israel) in 1/2× TBE) was loaded into reservoirs at the entrance of the nanochannels. An electric field was applied across the channels through electrodes immersed in the input and output reservoirs in a direction forcing the DNA into the channels. Once the DNA was stretched, the application of the voltage was stopped, and the stretched DNA was imaged.\(^{11,21}\)

**DNA Stretching on Modified Glass.** DNA molecules were stretched on glass coated with the hydrophobic polymer Zeonex to twice the B-form length, using DNA combing methods described by Neely et al.\(^{42}\) After stretching, the DNA was imaged using an epifluorescence microscope (FEI Munich GmbH, Germany) equipped with a high-resolution EMCCD IXon888 camera (Andor Technology Ltd., Belfast, U.K.). A 150 W xenon lamp (FEI Munich GmbH, Germany) was used for excitation with filter sets of 485/20ex and 525/30em and 650/13ex and 684/24em (Semrock Inc., Rochester NY, U.S.A.) for the YOYO-1 and Cy5 channels accordingly.

**SUPPORTING INFORMATION**
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**AUTHOR INFORMATION**
**Corresponding Author:** *E-mail: uv@post.tau.ac.il*

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