Alignment and Sensitive Detection of DNA by a Moving Meniscus

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In a process called "molecular combing", DNA molecules attached at one end to a solid surface were extended and aligned by a receding air/water meniscus and left dry on the surface. Extension by molecular combing of λ-DNA to $21.5 \pm 0.5\mu$m was observed. Using the combing process, we have further achieved (i) the extension of a 10^6 bp chromosomal Escherichia-coli DNA fragment and (ii) the sensitive detection of a minute quantity of DNA (10^3 molecules). These results open the way for a faster physical mapping of the genome and for the detection of small quantities of target DNA from a population of molecules.

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Extension and manipulation of individual biopolymers is generally performed by first anchoring one end of the molecule at a solid matrix; stretching may then be achieved by viscous drag [1] [2], electrophoretic [3] or optical forces [4]. The method proposed here, which we call " molecular combing," extends a DNA molecule with a receding meniscus and fixes the molecule in this state on the dry substrate. This physical process leads to a complete, controlled, and reproducible alignement of all DNA fragments, thus allowing accurate position determinations along the molecule.

To anchor DNA to a glass surface, a monolayer of silane molecules is first grafted onto a glass coverslip using methods of "molecular self-assembly" [5], exposing a vinyl end group ($-CH = CH_2$) [6]. These surfaces possess: (i) a high binding specificity only for the ends of a DNA molecule - presumably because of the presence of a free protonated phosphate at the 5' end - with a strong pH dependence, suggesting that the reaction between the molecules and the surface could be a case of electrophilic addition of weak acids to alkenes; (ii) the capability to bind proteins either directly or after oxidation to carboxylic acid groups; and (iii) a strong signal to noise ratio as a result of the negligible background fluorescence of glass. However, as usual with silanization procedures [7], the quality of the surface treatment is variable. As a result the percentage of anchored DNA molecules and their extension varies from batch to batch.

A drop of DNA solution (typically 5 μl) was deposited on a silanated coverslip. An untreated coverslip was then floated on top, forcing the drop to spread to a final thickness of $\sim 20\mu$m. With video enhanced fluorescence microscopy, molecules were observed [8] not only to be attached at one or both ends [9], as deduced from their extension by a flow or by electrophoresis but also to fluctuate freely in solution (Fig.1A), thereby indicating the absence of adhering non-specific interactions between the surface and the molecule.

During evaporation of the DNA solution, the receding air/water meniscus left the bound molecules fully extended behind and deposited on the dried surface, whereas unbound molecules were swept by the moving interface. The temporal extension of a single fragment of E-Coli DNA molecule by a receeding meniscus is shown in Fig.1(B-D) and can lead to the alignement of a 420μm long molecule [10] such as the one shown in Fig.1E. "Molecular combing" seems to be an irreversible process. Upon rehydration combed molecules remain bound to the surface.

It turns out that the force the meniscus exerts on the DNA is strong enough to extend it, but too weak to break the bond between the molecule and the surface. An estimate of the surface tension force on a rod of diameter D perpendicular to the surface of the meniscus is $F = \gamma\pi D$, where the surface tension $\gamma = 7\ 10^{-2}$ N/m for the air/water interface. Because $D = 2.2$ nm for the DNA diameter, $F \approx 4\ 10^{-10}$N. This force is 2 orders of magnitude greater than the entropic forces keeping the DNA molecule in a random coil configuration [2], and is thus enough to fully extend the molecule, but is apparently smaller than the force required to break a covalent bond (on the order of 10^{-9}N) [11]. Details on the physics of the combing process will be presented elsewhere. [12]

In contrast to viscous drag and electrophoresis, which act on the full length of the molecule, the action of the receding meniscus appears to be localized to the air/water interface, and is thus independent of the length and conformation of the molecule. In comparison to other techniques, the molecules are: (i) lying flat in a linearized state on a dry solid substrate; (ii) observable by fluorescence even several months after combing; and (iii) available for further manipulation. The local action of the meniscus is identical on all the molecules in solution: they are identically stretched. This is quantitatively shown by the distribution of lengths for a population of identical molecules which exhibits a well defined peak at $21.5 \pm 0.5\mu$m, see Fig.2. The "background noise" in that figure is mainly due to a random fragmentation of the DNA molecules. That fragmentation process is, we believe, a result of the dye induced fragilisation of the molecule and the shear forces [13] during handling and combing. Partial stretching can be ruled out since short segments are not noticeably more fluorescent that long ones.

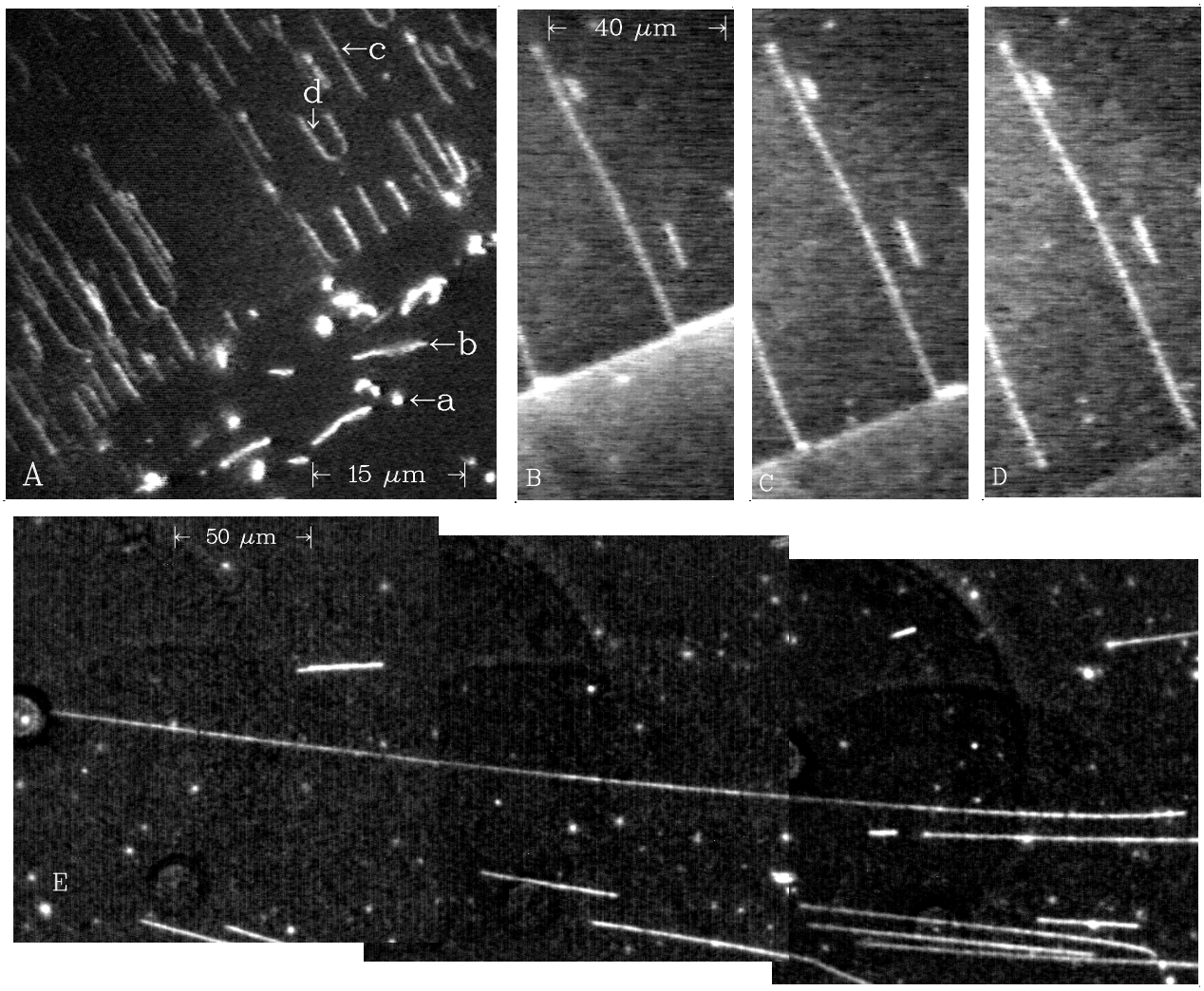

FIG. 1. Extension of DNA by a receding meniscus. (A) Video image showing $\lambda-$DNA in solution (lower right part of image), bound at one (a) or both extremities (b). The meniscus extends across the image from lower left to upper right. The extended molecules left behind the meniscus are visible as straight segments (c), if bound at one end, or loops (d), if bound at both ends. (B)-(D) Time series showing the extension of a chromosomal E.coli DNA fragment by a receding meniscus (lower part of the image). Time between the video images was 8.5 s. (E) Extension of an estimated 10^6 bp fragment of chromosomal E.coli, reassembled from 3 video images (total length= $420\mu m$).

TABLE I. Detection sensitivity of three concentrations of stained λ-DNA. The samples were manually and randomly scanned after combing. The deduced binding efficiency is $> 70\%$. This is a conservative estimate taking into account a possible 33% increase in the number of DNA segments due to fragmentation by shear, see Fig.2. A high binding efficiency is also consistent with the experimental observation that, while the meniscus receds, the number of swept unbound molecules is always much smaller than the number of combed ones.

Dilution # of mol.	# of fields of view*	Total DNA segments	Expected mean DNA / f.o.v	Sample mean DNA / f.o.v		std.error in the mean
10^5	77	397	5.2	5.16	$\pm$	0.32
10^4	70	48	0.52	0.69	$\pm$	0.09
10^3	122†	11	0.052	0.09	$\pm$	0.03

* One field of view (f.o.v.) $= 1.3\ 10^{-4}\mathrm{cm}^2$. Total sample area $= 2.5\mathrm{cm}^2$.

† This is a lower estimate because blank fields of view are difficult to count.

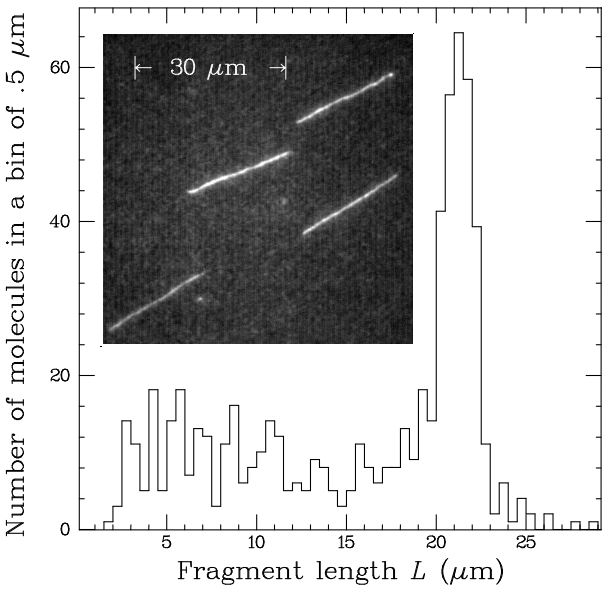

FIG. 2. Length distribution of extended $\lambda-$DNA on silanized surfaces, compiled from 111 video images, containing 635 DNA segments. The peak at $21.5\mu m$ (representing $\approx$ 50% of the molecules in the distribution) corresponds to a 33% increase over the unextended length of the molecule, 16.2μm. Inset: one of the 111 video images.

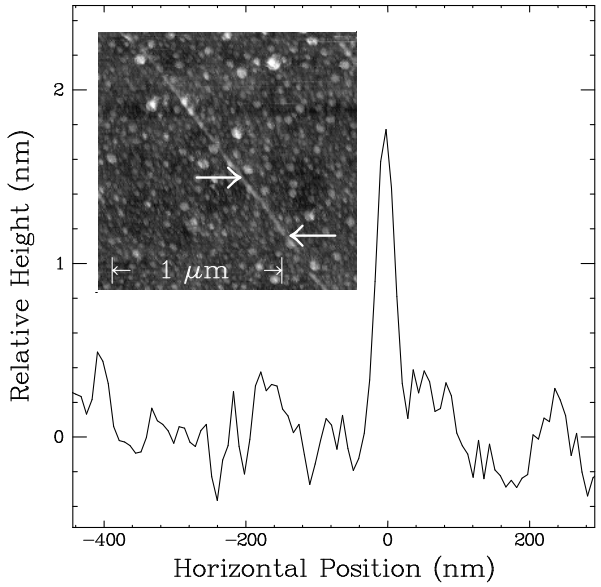

FIG. 3. Surface profile of a single combed DNA molecule scanned with an SFM. The noise in the SFM image is due to the roughness of the coverslip glass (typically $\pm 1.5nm$). In order to reduce that noise, the profile shown is an average over the profiles obtained from the 46 lines between the arrows in the AFM image shown in the inset.

It appears that DNA combing on silanized glass surfaces provides a direct method to prepare samples for structural studies with scanning force microscopy [14] [15] (SFM). From the averaged profile shown in Fig.3, we deduce that the diameter of a stained combed molecule is $1.8 \pm 0.8nm$ [16], consistent with the expected value for the width of a double helix ($2.4nm$ unstained) [17].

The dry stretched molecules are uniquely distinguishable from all other uncorrelated fluorescence. By scanning the sample, the number of extended molecules can be easily determined. To test the sensitivity of the technique, decreasing amounts of DNA (10^5, 10^4 and 10^3 molecules) in a volume of 5μl were loaded on silanized surfaces and then combed, see Table 1. From the observed density of molecules per unit area, one can estimate that more than 70% of the molecules can be combed, depending upon the quality of the surface treatment. Since the signal to noise ratio is independent of the number of molecules combed, the detection of a single molecule is only limited by the time required to scan the surface with the limited field of view of the objective. An attomole (10^{-18} moles = $6\ 10^5$ molecules) of DNA, is difficult to detect by methods not involving polymerase chain reaction (PCR) [18], but represents a huge number of molecules easily detectable by the method presented here. Other less sensitive methods use radioactive [19] and non-radioactive probes [20], with sensitivities of the order of 10^{-18} moles.

Silanized surfaces have been coated with proteins in order to increase the binding specificity of DNA to the surface. By treating a silanized coverslip, first with protein A and then with monoclonal anti-Digoxigenine [21], a surface is obtained which binds uniquely to end labelled Dig- dUTP λ-DNA (the rejection ratio between this Dig-labelled DNA and an unlabelled one is better than 10^5, data not shown). Furthermore, the anchored DNA (with an estimated better than 70% efficiency) could be combed, i.e. the Dig/anti-Dig bond is stronger than the tension exerted on the molecule by the receding meniscus [12].

Recently the introduction of direct visualization hybridization maps (DIRVISH) , which is an improvement of fluorescence in situ hybridization [22] (FISH), has demonstrated that high resolution multi color maps can be attained by cell lysis and subsequent hybridization [23]. Using molecular combing, a simple, controlled and reproducible optical mapping on purified DNA molecules (e.g. yeast artificial chromosomes) should be possible. After in vitro hybridization of DNA with probes stained with fluorescent antibodies or appropriate dyes and combing of the hybrid, it may be possible to measure distances with a resolution of 750 bp (250 nm) or better [24].

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[6] Trichlorosilanes ($SiCl_3 - (CH_2)_6 - CH = CH_2$) were reacted with the SiOH groups on coverglass, leaving the vinyl groups exposed. Coverglass surfaces were cleaned with a UV-ozone treatment[7], then transfered to a dessicator and flushed for 10 min. with Argon. Three hundred microliters of silane were added in a beaker near to the surfaces and left overnight to coat the coverglass. Immediately before use, the silanized surfaces were rinsed in de-ionized water and allowed to dry. λ DNA cl857 Sam7 (250 ng/μl) and bacterial chromosomal E.coli DNA (Strain GN48 in 1% low melting agarose plugs) were used. The E.coli DNA concentration was determined by optical density to be 3.6 ng/μl. Both DNA's were stained in a 5:1 or 10:1 ratio (bp per dye (YOYO-1) molecule). Immediately before use, stained DNA was diluted in a 50 mM Morpholino-ethane-sulfonic buffer (MES, pH 5.5) to a final concentration of 0.2 pM. Typically, 10 μl of that solution was loaded onto a silanized coverslip. An unsilanized, but well rinsed, coverslip then was floated gently on top and allowed to incubate at least 15 minutes at ambiant temperature and humidity before examination on the microscope. The binding of DNA on silanized surfaces was dependent on the pH: the number of bound molecules in $50mM$ MES buffer ($pH = 5.5$) is $\approx 10^4$ times greater than in 50 mM Tris buffer ($pH = 8.0$). Video images were taken on an inverted fluorescence microscope equiped with a 60x, 1.4 N.A. objective, 0.9-2.25x video zoom, 100 W Xenon source, optimized fluorescence filter set, and intensified CCD camera. Video signals were averaged (typically 16 frames) and contrast enhanced electronically by a commercial box and stored on tape. Images were digitized on a PC and analyzed with custom written software.

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[9] The probability of anchoring at both ends increases with incubation time.

[10] Note that this 1Mbp fragment of DNA is covalently bound to the silanized surface at only one of its 5' ends. All other methods we know of covalent (chemical) attachment of oligonucleotides to a surface lead to multiple parasitic bindings, see V. Lund, R. Schmid, D. Rickwood, E. Hornes, Nucl. Acids Res. **16**, 10861 (1988).

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[13] For a molecule of length L, the shear force is $F \approx \eta L v$, where η is the viscosity (10^{-2} poise for water). Taking a lower estimate for the rupture force to be $\approx 10^{-9}$N, shear velocities of a few hundred μm/sec are enough to break an E-Coli chromosomal DNA.

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[15] Stained λ DNA (7 10^6 molecules) was loaded onto a silanized surface and observed as described in Fig. 1. They were then imaged with a scanning force microscope, in immersion oil with a standard pyramidal tip V-shaped cantilever ($100\mu m$ long, $0.6\mu m$ thick) with spring constant $k = 0.21$ N/m. The piezoelectric tube has a $75\mu m$ scanning range. The image shown is a part of a $4\mu m$ by $4\mu m$ scan with 512 by 512 pixels. It was taken in topographic mode, at a 5 Hz line frequency.

[16] From the full width at half maximum of the profile, one estimates the lateral resolution to be of order of $30nm$, 10 times better resolved than standard optical microscopy. Furthermore, we have verified that the DNA was fully linearized without knots by screening the length of a molecule with the SFM.

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[21] The right cos-end of λ-DNA was 3' end labelled with Dig-dUTP by terminal transferase and ligated to λ-DNA. Silanized surfaces were treated with 60μl of 1 mg/ml Protein-A in water for 2 hours at room temperature, rinsed three times in de-ionised water and then incubated with 60μl of 0.1 mg/ml anti-Dig solution for 2 hours at room temperature. Surfaces where then rinsed three times in de-ionised water. 4μl of YOYO1 stained molecules (1.6 pM in 10 mM Tris, pH = 8) were deposited on these prepared surfaces, covered with a rinsed unsilanized coverslip and left to incubate in a humid atmosphere at room temperature for 1 hour, then left to dry.

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