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- (54) **METHOD OF DNA ANALYSIS USING MICRO/NANOCHANNEL**
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7,005,264	B2 *	2/2006	Su et al.	435/6
7,217,562	B2 *	5/2007	Cao et al.	435/287.2
2002/0119455	A1 *	8/2002	Chan	435/6
2002/0187505	A1	12/2002	Stockton	
2002/0187508	A1	12/2002	Wong	
2003/0022317	A1 *	1/2003	Jack et al.	435/91.1
2003/0044777	A1 *	3/2003	Beattie	435/6
2003/0059822	A1 *	3/2003	Chan et al.	435/6
2003/0100094	A1 *	5/2003	Heiter et al.	435/199
2004/0033515	A1 *	2/2004	Cao et al.	435/6
2004/0195098	A1 *	10/2004	Broadley et al.	204/435
2004/0197843	A1 *	10/2004	Chou et al.	435/7.92
2005/0221507	A1 *	10/2005	Koo et al.	436/525
2006/0063171	A1 *	3/2006	Akeson et al.	435/6
2006/0068440	A1 *	3/2006	Chan et al.	435/6
2006/0286570	A1 *	12/2006	Rowlen et al.	435/6
2007/0009954	A1 *	1/2007	Wang et al.	435/6
2007/0031829	A1 *	2/2007	Yasuno et al.	435/6
2007/0042400	A1 *	2/2007	Choi et al.	435/6
2007/0042419	A1 *	2/2007	Barany et al.	435/6

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(51) **Int. Cl.**

C12Q 1/68 (2006.01)
C12M 1/34 (2006.01)
C07H 21/04 (2006.01)
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(52) **U.S. Cl.** **435/6; 435/287.2; 536/23.1; 436/800**

(58) **Field of Classification Search** None
See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

5,200,313	A *	4/1993	Carrico	435/6
5,230,866	A *	7/1993	Shartle et al.	422/103
6,713,263	B2 *	3/2004	Schwartz	435/6

OTHER PUBLICATIONS

Heng, et al. *Biophysical Journal*, Oct. 2004; 87:2905-2911.*
Zhang et al., *Bioinformatics*, 2003, vol. 19, No. 1, pp. 14-21.*
New England Biolabs, Nt.BbvCI webpage, Aug. 12, 2008.*
Bousse L, et al., "Electrokinetically controlled microfluidic analysis systems," *Annu. Rev. Biophys. Biomol. Struct.* 29:155-181 (2000).
Helter D, et al., "Site-specific DNA-nicking mutants of the heterodimeric restriction endonuclease R.BbvCI," *J.Mol. Biol.* 348:631-640 (2005).
Rye J, et al., "Stable fluorescent dye-DNA complexes in high sensitivity detection of protein-DNA interactions Applications to heat shock transcription factor," *Nucl Acids Res*, 20(11):2803-2812 (1993).
Wabuyele B, et al., "Single molecule detection of double-stranded DNA in poly(methylmethacrylate) and polycarbonate microfluidic devices," *Electrophoresis* 22:3939-3948 (2001).
Roberts, Richard J., A nomenclature for restriction enzymes, DNA methyltransferase, homing endonucleases and their genes, *Nucleic Acids Reseach* 2003, vol. 31, No. 7, 1805-1812.
Samuelson, James C., The isolation of strand-specific nicking endonucleases from a randomized SapI expression library, *Nucleic Acids Research* 2004, vol. 32, No. 12, 3661-3671.

* cited by examiner

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(57) **ABSTRACT**

Methods are provided for tagging, characterizing and sorting double-stranded biomolecules while maintaining the integrity of the biomolecules.

12 Claims, 4 Drawing Sheets

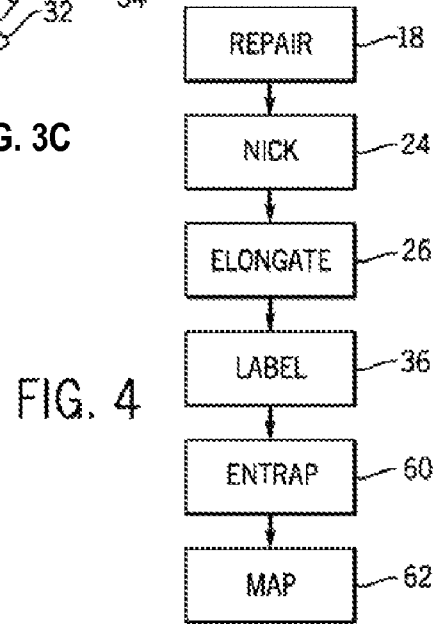
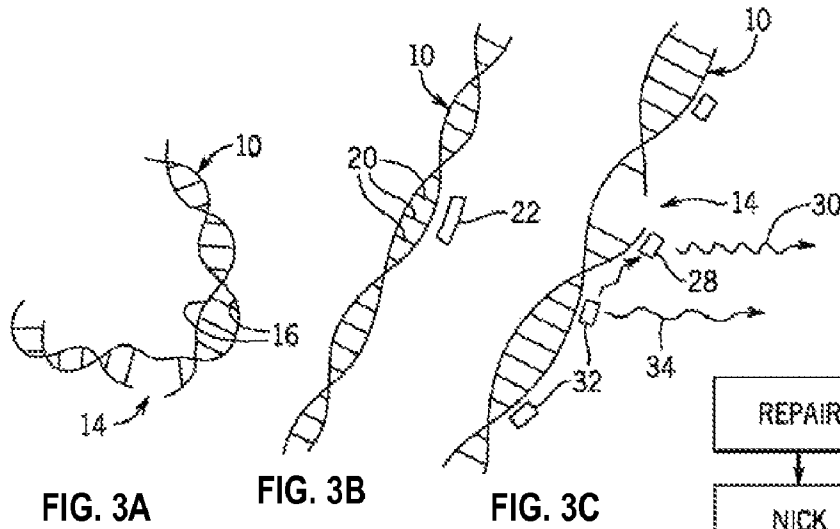
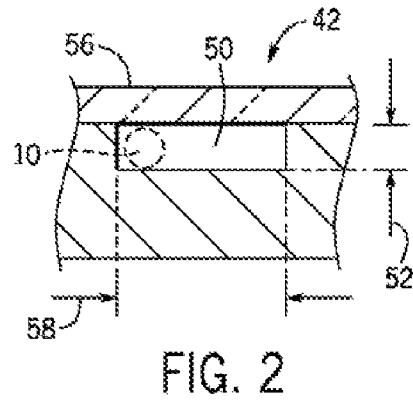
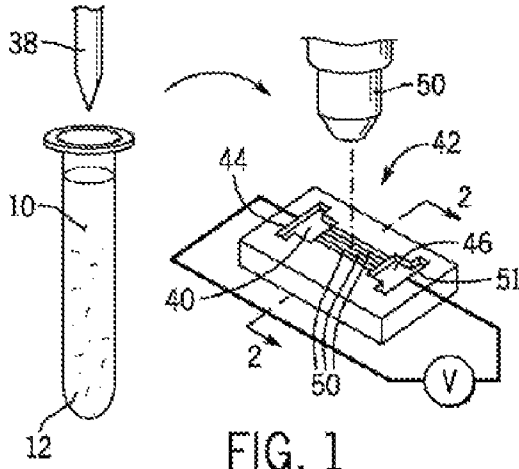


FIG. 5A

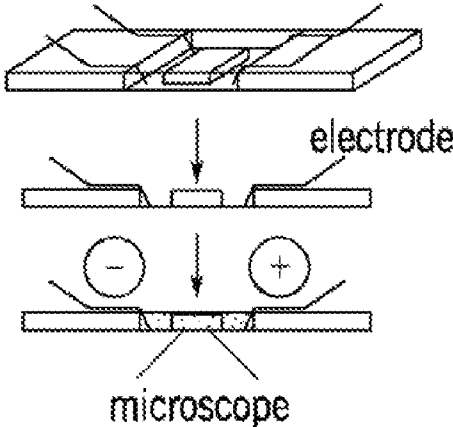


FIG. 5B

nanoslit

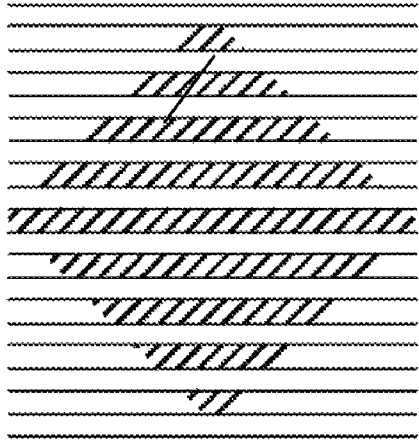


FIG. 6A

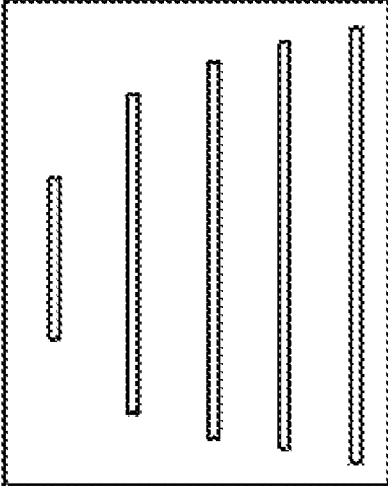
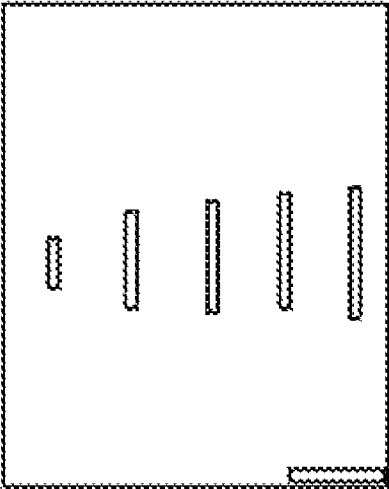


FIG. 6B



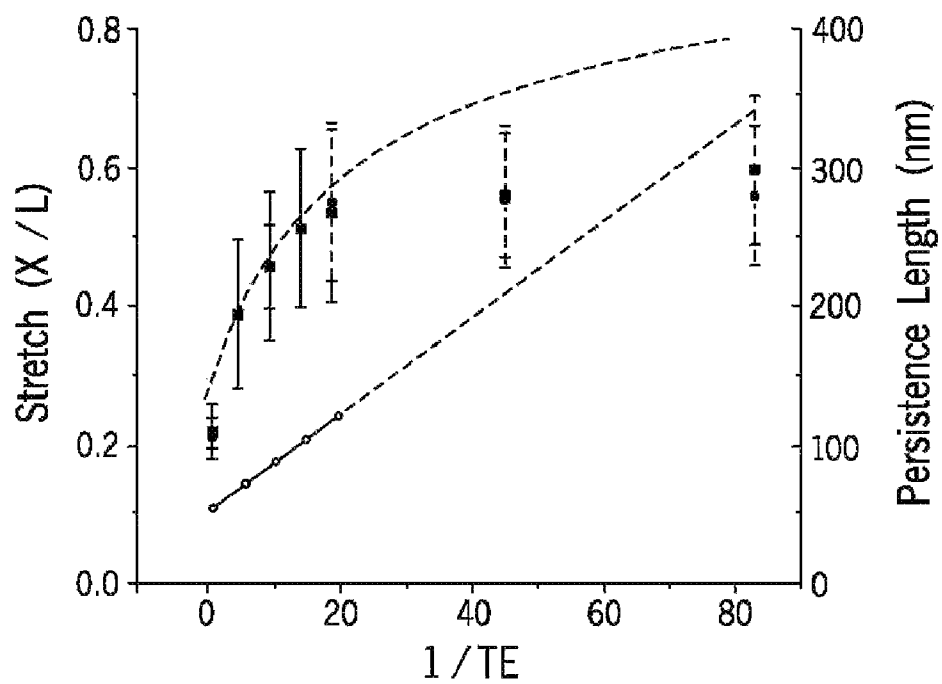


FIG. 7

METHOD OF DNA ANALYSIS USING MICRO/NANOCHANNEL

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Patent Application Nos. 60/740,583, filed Nov. 29, 2005; and 60/740,693, filed Nov. 30, 2005, each of which is incorporated herein by reference as if set forth in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with United States government support awarded by the following agencies: NIH HG000225 and NSF 0425880. The United States has certain rights in this invention.

BACKGROUND OF THE INVENTION

The present invention relates to methods of presenting polymeric biomolecules such as nucleic acids for analysis, and in particular, to methods for tagging and elongating the biomolecules for characterization, sorting and analysis or other use.

A single DNA molecule can be elongated, for example, by shear forces of liquid flow, by capillary action or by convective flow, and then fixed in an elongated form to a substrate by electrostatic attraction. Sequence features of the fixed molecule can be identified by cleaving the fixed molecule with one or more restriction enzymes to produce gaps that can be marked by fluoroscopic markers or the like and then visualized. See, e.g., U.S. Pat. Nos. 6,713,263; 6,610,256; 6,607,888; 6,509,158; 6,448,012; 6,340,567; 6,294,136; 6,221,592. See also, U.S. Published Patent Application Nos. 2005/0082204; 2003/0124611; 2003/0036067. Each patent and published patent application is incorporated herein by reference as if set forth in its entirety. Although fixing the DNA to the substrate simplifies the analysis by preserving the elongated state, stabilizing the position of the molecule, and preventing fragment shuffling after cleavage, it is difficult to control chemical interactions with the DNA because of its close proximity to the substrate. Also, cleaved DNA may not be suited for subsequent use, especially where fragment shuffling diminishes information content that can be obtained from an uncleaved molecule.

One can avoid such surface effects by suspending the DNA molecule in a "nanochannel" without attachment to the channel walls. A nanochannel is a channel having a cross-sectional dimension less than 1,000 nanometers and typically on the order of 30 nanometers. The suspended DNA molecule is sufficiently spaced apart from the channel walls to avoid surface interference in the reaction process while still stabilizing the DNA molecule sufficiently for analytical techniques.

Practical use of nanochannels faces a number of obstacles. Nanochannels are extremely difficult to fabricate, thus costly, and as a practical matter, are not reusable. In addition, the small size of nanochannels makes the addition of chemical reagents, especially enzymes, difficult. It is also difficult to encourage DNA molecules to enter the small cross-sectional area of nanochannels. Likewise, it can be difficult to remove interfering reaction by-products from nanochannels. Finally, because DNA is not under significant tension within nanochannels, restriction enzymes cutting the DNA may not make visible gaps.

SUMMARY OF THE INVENTION

The present invention provides methods for marking individual double-stranded polymeric nucleic acid molecules to yield intact, marked polymeric nucleic acid molecules suitable for characterization and for subsequent uses. In accord with the method, a single strand of the double stranded molecule is broken (nicked) but the integrity of the molecule is maintained. Because the molecule is not cleaved in the nicking process, the nicking process can be performed before the molecule is immobilized for analysis in a nanochannel, and optionally characterized and sorted for subsequent uses, including use in an array of sorted molecules.

The invention also relates to methods for providing the nicked, polymeric nucleic acid molecules in a low ionic strength buffer to increase the stiffness of the molecules, thereby facilitating use of devices having convenient geometries for characterization.

The third technique, rendered possible by the increased stiffness, is to use a channel having a nanometer scale in height, but a micrometer scale in width, relying on the greater stiffness of the elongated DNA molecule to remain properly aligned within the larger channels. The larger channel simplifies loading of the DNA, the introduction of reagents, and greatly simplifies channel construction, thereby reducing costs and making disposable channels practical.

These simplified processes for preparing the individual DNA molecules (which can include multiple copies of particular individual molecules, where, e.g., multiple genome-equivalents are analyzed) away from a surface and before introduction into the nanochannel, also simplify marking of the DNA molecule with multiple fluoroscopic materials. The use of fluorescence resonance energy transfer (FRET), in which one fluorescent material produces light exciting the other, allows dual color imaging and reduces the effects of unincorporated dyes because of the limited range of this effect. The profile of fluorescent labels on each imaged molecule, referred to as its 'barcode,' is characteristic of that molecule. From the characteristic barcode, the location of the molecule can be ascertained from an existing database of nucleic acid sequences of the source organism, tissue or cell type, in a manner known to the art. Individual molecules of interest can be flagged according to a user-specified set of parameters for further collection, analysis or other use.

In another aspect, the invention relates to sorting of nucleic acids characterized in accord with the invention. In particular, a nucleic acid molecule characterized in a nanochannel after site-specific labeling on a single strand as described elsewhere herein can be captured by, e.g., electrostatic attraction, to an electrode activated to capture on the particular molecule (or, e.g., a related class of molecules). The electrode can be provided in a microchannel (i.e., a channel having width and height dimensions greater than those of a nanochannel, e.g., 5 microns in width and 10 microns in height) in electrical connection with and under the logical control of a controller (such as a computer having a suitable user interface for specifying user parameters) that can also coordinate the process of matching the labeled nucleic acids against a genomic sequence database to identify and distinguish nucleic acid molecules of higher interest from those of lesser interest. In turn, the controller can address the electrode independently, thereby facilitating separate collection of one or more molecules at defined positions in an array. A plurality of electrodes, each under separate or coordinated control of one or more controllers, can effectively capture and accumulate a plurality of identical, related or distinct molecules characterized as described. Electrodes can be provided in a matrix

suited for electrophoretic migration of nucleic acids, e.g., agarose or polyacrylamide, such that captured molecules tend to remain at or near the electrode until directed by the controller to release the captured molecules. Alternatively or additionally, a ligand can be provided at the capture site to fixedly or releasably retain the captured molecules at the site. A suitable capture system can be based upon the addressable electrode arrays described in various patents assigned to Nanogen, Inc., such as U.S. Pat. No. 7,101,717; U.S. Pat. No. 7,045,097; and U.S. Pat. No. 6,867,048, each of which is incorporated herein by reference as if set forth in its entirety. Other systems for capturing and/or releasing nucleic acid can be envisioned.

After capture on one or a plurality of such electrodes, identical or related captured molecules can be released from the electrodes in an ordered manner by reversing charge on the electrodes. Again, the release can be separately or coordinately controlled by the controller or controllers. Upon release, the molecules can be conveyed for subsequent analysis, such as sequencing analysis. The powerful labeling and identification features of the invention, coupled with the ability to selectively capture or discard identified molecules, yield a system for high-throughput analysis of nucleic acid molecules. One appropriate use for such released molecule(s) is a conventional nucleotide-level sequence analysis of the molecules to ascertain whether the obtained molecule(s) are identical to or different from a reference sequence in a pre-existing database. In a related embodiment, after the profile of each molecule is determined in a nanochannel and its value assessed, the molecule flows in a stream from the nanochannel into a sorting microchannel-sized chamber whereupon the controller instructs a microvalve in the chamber either (1) to open to reject a molecule of low interest from the chamber into a waste chamber or (2) to close so that a molecule of high interest is drawn (e.g., by charge) into a downstream system, such as a sequencing platform or amplification system. It will be understood that high throughput can be achieved in any of these embodiments by providing a plurality of nanochannels for sorting multiple molecules in parallel from a single inlet source along with a controller network and software having adequate facility to manage the parallel sorting and disposition of the tagged molecules.

These and other features, aspects and advantages of the present invention will become better understood from the description that follows. In the description, reference is made to the accompanying drawings, which form a part hereof and in which there is shown by way of illustration, not limitation, embodiments of the invention. The description of preferred embodiments is not intended to limit the invention to cover all modifications, equivalents and alternatives. Reference should therefore be made to the claims recited herein for interpreting the scope of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will be better understood and features, aspects and advantages other than those set forth above will become apparent when consideration is given to the following detailed description thereof. Such detailed description makes reference to the following drawings, wherein:

FIG. 1 is a graphical representation of the process of the present invention in which DNA can be chemically manipulated before being inserted in a nanochannel for optical mapping;

FIG. 2 is a cross-sectional view along line 2-2 of FIG. 1 through a nanochannel having nano- and micro-scale features;

FIGS. 3*a*, 3*b* and 3*c* are simplified representations of DNA during three stages of the present invention in which the DNA is repaired, nicked, elongated, labeled and entrapped within a nanochannel;

FIG. 4 is a flow chart showing these principle steps of the present invention; and

FIGS. 5*a* and *b* depicts a design of a suitable micro-nanochannel device.

FIGS. 6*a* and *b* show the effect of buffer ionic strength on DNA elongation.

FIG. 7 graphically depicts the impact of buffer dilution upon DNA elongation.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The present invention relates to the observation that when large genomic DNA molecules are a primary analyte, a key issue becomes how to unravel and mark DNA molecules for high-throughput application. With respect to unraveling DNA molecules, high-throughput methods and systems using nanochannels for analyzing single, intact DNA molecules are possible at low buffer ionic strength because persistence length of DNA molecules inversely varies with ionic strength of a buffer. Likewise, and with respect to marking of DNA molecules, labeled, sequence-specific nucleotides can be added to DNA molecules nicked at specific sites using endonucleases that do not catalyze double strand cleavage of the molecules, thereby providing markers of site-specific nicking. Using available genomic sequence information, one can correlate the site-specific markers with identifiable nucleic acid molecules. Desired nucleic acid molecules so identified can be sorted and collected for subsequent use. It will be appreciated that intact individual nucleic acid molecules prepared for presentation and analysis as described herein can alternatively be analyzed after being deposited on a surface, with the understanding that difficulty in removing such deposited molecules precludes subsequent analysis of such molecules, e.g. by nucleic acid sequencing or other methods.

Referring to FIG. 1, per the present invention, DNA 10 can be initially processed in a solution 12 without being affixed to a surface such as may interfere with some chemical reactions. For example, a DNA 10 can be dyed with a fluorescent marker, such as YOYO-1 fluorescent dye (hereinafter, "YOYO-1").

Referring also to FIGS. 3*a* and 4, DNA 10 in solution, however, may include a number of inherent or pre-existing nicks 14 in which one strand 16 is broken. Such nicks 14 may result from damage from mechanical damage, UV light, or temperature.

In a first step, as represented by process block 18 of FIG. 4, incidental inherent nicks 14 are repaired to prevent confusion with nicks made for marking purposes. That is, DNA ligase and dideoxy nucleotides are added to the DNA 10 in solution to remove the inherent nicks. In addition, or alternative, a nuclease-free DNA polymerase (such as DNase-free DNA Polymerase I, available from Roche Applied Science) and dideoxy nucleotides are added to the DNA 10 in solution.

As shown in FIG. 3*b*, and represented by process block 24 of FIG. 4, the suspended DNA 10, as repaired, can be nicked at predefined base pair sequences 20 by enzyme 22. For example, the enzyme 22 can be a genetically engineered nicking endonuclease that cleaves only a single strand of a double-stranded polynucleotide, such as an enzyme of this class commercially available from New England Biolabs, including Nb.BbvCI and others. Typically, 20 units of Nb.BbvCI and deoxynucleotides are allowed to incubate with

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DNA **10** in solution at 37° C. As indicated by FIG. 3c, and represented by process block **36**, the nicks **14** produced by enzyme **22** can be labeled by incorporating at the nicked site at least one fluorochrome **28** labeled deoxynucleotide providing a given frequency of light emission **30**, and the remaining body of the DNA **10** can be labeled with a second fluorochrome **32** having a second frequency of emission **34**. Fluorochrome **28** can be uniquely keyed to the point of the nick **14** whereas the fluorochromes **32** can distribute themselves uniformly over the remaining surface of the DNA **10**. The nicked, labeled DNA can thus be imaged using Fluorescence Resonance Energy Transfer. These labeled nicks, when identified in position, will reveal characteristic sequence properties of the DNA **10**.

Per process block **26** of FIG. 4, salt then can be removed from the DNA **10** by adjustment of the buffering solution **12** to cause an elongation of the DNA **10** and a corresponding increased rigidity.

The elongated and labeled DNA in solution may then be removed for example, by a pipette **38** and transferred into a first chamber **40** of a nanochannel assembly **42**. The first chamber **40** provides one electrode **44** of an electrophoresis device and communicates through a set of nanochannels **50** with a second chamber **46** of the assembly **42** having a second electrode **51** of the electrophoresis device. As will be understood in the art, operation of a voltage across electrodes **44** and **51** will draw charged molecules such as DNA **10** from first chamber **40** to second chamber **46** through the nanochannels **50** extending therebetween. This step entraps the DNA within the nanochannels **50** for analyses or subsequent processing, as represented by process block **60** of FIG. 4.

Referring to FIG. 2, each nanochannel **50** may have a height **52** on the order of 30 nanometers effectively constraining the DNA **10** from substantial curvature in a vertical plane based on the cross-sectional diameter of the DNA **10**. This constraint ensures that the DNA is maintained within the focal plane of a microscope objective which may view the DNA **10** through a transparent top wall **56** of the nanochannels **50**.

The width of the channel **58** can be on the order of 1,000 nanometers (one micrometer) or less. This allows for simple fabrication of the channel using elastomeric molding techniques, for example, and improves the ability to draw the DNA into the nanochannels **50**. The increased stiffness of the DNA **10** preserves its orientation and alignment in the nanochannels **50** despite the width of the nanochannels **50**.

The DNA **10** may then be characterized or manipulated in other ways within the nanochannels **50** per process block **62**. One wall of the nanochannels **50** can be semi-permeable to perform reactions on or otherwise affect the DNA **10**, for example, restoring salt to the DNA.

Upon exit from the nanochannel, characterized molecules can be sorted or selected on the basis of user-specified parameters, as described. For example, the molecules can be sorted onto an array on the basis of chromosomal heritage (i.e., only DNA from a particular chromosome or set of chromosomes can be retained, if desired, by providing appropriate capture ligands. Alternatively, related sequences of, e.g., DNA encoding closely related genes can be retained with each capture ligand retaining a single unique version of the gene. Similarly, members of a single gene family can be sorted away from other related or unrelated genes. Also, molecules evidencing known aberrations or mutations can be sorted for further analysis. It will be apparent that the sorting possibilities are extensive.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood

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by one of ordinary skill in the art to which the invention belongs. Although any methods and materials similar to or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described herein.

As used herein, persistence length refers to basic mechanical property quantifying the stiffness of a macromolecule of a polymer, reflecting the relative directional orientation of several infinitesimal segments. Under common laboratory conditions, DNA molecules have a persistence length of ~50 nm.

Example 1

Construction of Nanochannel Devices

Fabrication of nanochannel devices followed standard soft lithography techniques known to one skilled in the art. Whitesides G, et al., "Soft lithography in biology and biochemistry," *Annu. Rev. Biomed. Eng.* 3:335-373 (2001). To begin, a chrome mask, created with e-beam lithography at the University of Wisconsin Center for nanotechnology, was used as a mask in photolithography on negative photoresist spin-coated onto silicon wafers, creating an array of 1 μ m channels. The wafer was etched to a depth of 100 nm by CF₄ gas. Photoresist was lifted off by piranha solution. The height of the pattern was determined by an alpha step profilometer and the width of the pattern was measured under scanning electron microscope. After fabrication of the nanochannels, a microchannel array was overlaid on the nanopatterned wafer. SU-8 2005 photoresist was used to create channels 5 μ m high and 100 μ m wide. FIG. 5 shows the nanochannels (diagonal lines; 100 nm high \times 1 μ m wide) overlaid with microchannels (horizontal lines; 3 μ m high \times 100 μ m wide).

Next, the devices were formed by molding polydimethylsiloxane (PDMS; DOW CORNING; Midland, Mich.) onto the wafer described above. PDMS replicas were formed by curing the PDMS for 24 hours at 65° C. A short curing time (i.e. less than 24 hours), is not enough to make stable, PDMS nanochannel devices. An O₂ plasma treatment (O₂ pressure of ~0.67 millibars; load coil power 100 W; 36 seconds; Technics Plasma GMBH 440; Florence, Ky.) of the PDMS nanochannel devices was used to render the devices hydrophilic. Plasma-treated devices were stored in ultrapure water for twenty-four hours, as PDMS surfaces are reactive right after plasma treatment. Long-time storage in pure water makes the surfaces less reactive. In addition, the PDMS nanochannel devices were washed with 0.5 M EDTA (pH 8.5) three times to extract platinum ions, which was a catalyst for PDMS polymerization. Finally, the PDMS nanochannel devices were mounted on acid cleaned glass, prepared as described previously. Dimalanta E, et al., "A microfluidic system for large DNA molecule arrays," *Anal. Chem.* 76:5293-5301 (2004).

Example 2

Sequence-Specific Labeling of Nicked DNA

Sequence-specific labeled DNA was the result of the following steps: (1) linearization, (2) ligation, (3) nick site blocking, (4) nick translation, and (5) protein digestion. Linearization is required only when using circular DNA. Regardless of whether linearization was required, DNA ligase was used to remove inherent nicks in DNA. A DNA ligase reaction with T4 DNA ligase was performed at room temperature overnight. An overnight reaction ensures that ligation is complete and kills DNA ligase activity. To further ensure that and

all inherent nicks were removed, DNA polymerase I (10 units) was added with dideoxy nucleotides (ddNTPs; 0.2 μM each) for 30 minutes at 37° C. ddNTPs incorporated into nicks block additional polymerase reaction and avoid random labeling. A nicking enzyme (Nb.BbvCI, 20 units; GC⁺T-GAGG) was then added with deoxynucleotides (dNTPs), such as ALEXA FLUOR 647-aha-dCTP fluorescent marker (2 μM), ALEXA FLUOR 647-aha-dUTP fluorescent marker (2 μM), dATP (20 μM), dCTP (1 μM), dGTP (20 μM) and dTTP (1 μM). Because DNA polymerase was added previously, no additional DNA polymerase was added during nick translation. The nick translation reaction was performed for 30 minutes at 37° C. EDTA (pH 8.0, 20 μM) was added to quench reactions. Proteinase K (100 ng/μl) and lauroyl sarcosine (0.1% w/v) were added to remove all enzymes.

Following sequence-specific labeling, DNA was counterstained with YOYO-1 (25 μM; Molecular Probes; Eugene, Oreg.). DNA base pairs to YOYO-1 in a ratio of 6:1 for λ DNA and a ratio of 5:1 to T4 DNA. Final DNA samples containing DNA (1 ng/μl of λ DNA or 0.78 ng/μl of T4 DNA), Tris-EDTA buffer (pH 8.0), β-mercaptoethanol and POP6 (0.1% w/v; APPLIED BIOSYSTEMS; Foster City, Calif.) were then loaded into the microchannels by capillary action and subsequently entered the nanochannels by application of an electric field. Tris-EDTA buffer concentration varied from 1×TE (10 mM Tris and 1 mM EDTA) to 0.01×TE (100 μM Tris and 10 μM EDTA). The ionic strength of the buffer can be varied both before or after entry into the nanochannels to affect elongation of the DNA.

An argon ion laser-illuminated inverted ZEISS 135M microscope was used to image DNA molecules. The microscope was equipped with a 63× ZEISS Plan-Neofluar oil immersion objective, a DAGE SIT68GL low-light level video camera connected to a SONY monitor for visual inspection of DNA molecules, and a charge-couple device (CCD) camera for acquiring focus and high-resolution images. A LUDL ELECTRONICS x-y stage and focus motor with 0.1-m resolution was used for x-y-z translation. Two emission filters were installed in the microscope, YOYO-1 emission filter XF3086 and ALEXA FLUOR 647 emission filter. The YOYO-1 filter was used to take images of DNA backbones; whereas the ALEXA FLUOR 647 filter was used to take fluorescence resonance energy transfer (FRET) images.

Example 3

Elongation of Labeled DNA

As shown in FIGS. 6a and 6b, diminished ionic strength buffer conditions increased intrachain electrostatic repulsion of DNA in the nanochannels, thereby increasing persistence length. Specifically, FIGS. 6a and 6b show typical images of T4 DNA (166 kbp; polymer contour length, 74.5 μm) and λDNA (48.5 kbp; polymer length, 21.8 μm) elongated within nanochannels under low ionic strength buffer conditions (0.05×TE and 0.01×TE) showing apparent lengths of 40.9±8.4 μm (T4 DNA) and 13.0±2.4 μm (λDNA).

As shown in FIG. 7, DNA elongation is a function of the dilution of TE (1/TE) reported as "stretch," and defined as the average apparent length, X, divided by the dye adjusted polymer contour length, L (e.g., L=21.8 for λDNA); a stretch value of 1.0 indicates complete elongation. FIG. 7 also shows that the degree of DNA elongation is size independent and inversely related to salt concentration or ionic strength used for the calculation of persistence by the following equation:

$$P = P_o + P_{ei} = P_o + 1 / (4\kappa^2 l_b) = P_o + 0.324 l^{-1} \text{ \AA};$$

where P_o is the non-electrostatic intrinsic persistence length due to base stacking, P_{ei} is the electrostatic persistence length due to intrachain repulsion, κ^{-1} is the Debye-Hückel screening length, and l_b is the Bjerrum length (7 Å in water).

The invention has been described in connection with what are presently considered to be the most practical and preferred embodiments. However, the present invention has been presented by way of illustration and is not intended to be limited to the disclosed embodiments. Accordingly, those skilled in the art will realize that the invention is intended to encompass all modifications and alternative arrangements within the spirit and scope of the invention as set forth in the appended claims.

We claim:

1. A method of analyzing a double-stranded genomic DNA molecule for a sequence structure, the method comprising the steps of:

- (a) introducing a sequence-specific nick at each recognition site on at least one strand of the DNA molecule using a nicking endonuclease under nicking conditions creating a pattern of nicks characteristic of the DNA molecule, wherein the molecule maintains its integrity;
- (b) labeling each nicked recognition site with a first label;
- (c) labeling a remainder of the DNA molecule with a second label;
- (d) elongating the DNA molecule;
- (e) passing the labeled DNA molecule into a channel to hold the DNA molecule in an elongated state; and
- (f) detecting the first label to identify the pattern of nicks of the DNA molecule revealing the sequence structure.

2. The method of claim 1, wherein the nicking endonuclease is Nb.BbvCI.

3. The method of claim 1, wherein the first label is a fluorescent molecule.

4. The method of claim 1, wherein the second label is a fluorescent molecule.

5. The method of claim 4, wherein the fluorescent molecule is YOYO-1.

6. The method of claim 1, wherein the DNA is held in suspension within the channel.

7. The method of claim 1, the method further comprising the step of repairing endogenous nicks prior to sequence-specifically labeling the genomic DNA molecule.

8. A method of determining a sequence structure of double-stranded genomic DNA comprising the steps of:

- (a) marking a body of the genomic DNA molecule suspended in a solution with a fluorescent marker not specific to particular features on the DNA molecule;
- (b) marking sequence-specific positions of the genomic DNA molecule with a different fluorescent marker by nick translating the molecule from a sequence-specific nick introduced with a nicking endonuclease that does not catalyze double strand cleavage, wherein the molecule maintains its integrity and wherein the marked sequence-specific positions define a pattern characteristic of the DNA molecule;
- (c) elongating the genomic DNA molecule;
- (d) exciting the marked DNA molecule with a light source to distinguish a difference in color between the fluorescent markers to reveal the pattern; and
- (e) detecting the pattern characteristic of the DNA molecule to determine the sequence structure of the genomic DNA molecule.

9. The method of claim 8, wherein the fluorescent markers are fluorescence resonance energy transfer markers.

10. A method for sorting individual nucleic acid molecules, the method comprising the steps of:

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- (a) introducing sequence-specific nick sites into each molecule with a nicking endonuclease that does not catalyze double strand cleavage, wherein the molecule maintains its integrity;
- (b) incorporating at the sequence-specific nick sites a label to yield labeled nick sites that mark the nucleic acid molecules and thereby define a pattern characteristic of each marked nucleic acid molecule;
- (c) elongating the nucleic acid molecules;
- (d) passing the marked nucleic acid molecules into a channel holding the nucleic acid molecules in an elongated state;
- (e) correlating the pattern of each marked nucleic acid molecule known nucleic acid sequences;

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- (f) passing the marked nucleic acid molecules from the channel; and
 - (g) capturing the marked nucleic acid molecules.
11. The method as claimed in claim 10, wherein the step of capturing the marked DNA molecule includes the steps of activating a charge-based capture pad.
12. The method as claimed in claim 11, wherein the method further comprises the steps of conveying analytical information about the marked molecule to a controller and selectively activating the charge-based capture pad depending upon the identity of the marked molecule.

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