Combed Single DNA Molecules Imaged by Secondary Ion Mass Spectrometry

ARTICLE *in* ANALYTICAL CHEMISTRY · AUGUST 2011

Impact Factor: 5.64 · DOI: 10.1021/ac201685t · Source: PubMed

analytical chemistry

ACCELERATED ARTICLE

pubs.acs.org/ac

Combed Single DNA Molecules Imaged by Secondary Ion Mass Spectrometry

Armelle Cabin-Flaman,[†,‡] Anne-Françoise Monnier,[†,§] Yannick Coffinier,[∥] Jean-Nicolas Audinot,[⊥] David Gibouin,[†,‡] Tom Wirtz,[⊥] Rabah Boukherroub,[∥] Henri-Noël Migeon,[⊥] Aaron Bensimon,[⊗] Laurent Jannière,[‡,§] Camille Ripoll,[†,‡] and Vic Norris*[,†,‡]

[†]Equipe Assemblages Moléculaires: Modélisation et Imagerie SIMS, Laboratoire MERCI EA 3829, Faculté des Sciences de l'Université de Rouen, 76821 Mont Saint Aignan Cedex, France

[‡]Epigenomics Project, Genopole, 91000 Evry, France

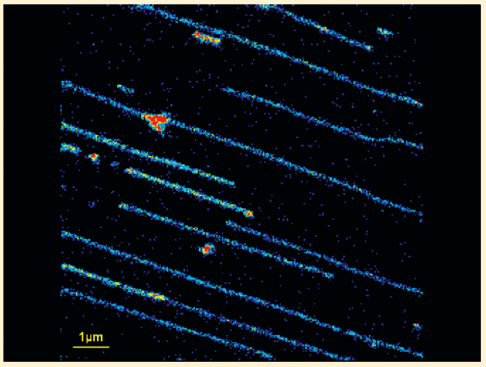
[§]Laboratoire de Génétique Microbienne, INRA, Domaine de Vilvert, 78352 Jouy-en-Josas, France

[∥]Institut de Recherche Interdisciplinaire (IRI, USR-3078) and Institut d'Electronique, de Microélectronique et de Nanotechnologie (IEMN, CNRS-8520), Cité Scientifique, Avenue Poincaré—B.P. 60069, 59652 Villeneuve d'Ascq, France

[⊥]Département Science et Analyse des Matériaux, Centre de Recherche Public Gabriel Lippmann, 4422 Belvaux, Luxembourg

[⊗]Genomic Vision, 27 Rue du Faubourg Saint Jacques, Paris 75014, France

ABSTRACT: Studies of replication, recombination, and rearrangements at the level of individual molecules of DNA are often limited by problems of resolution or of perturbations caused by the modifications that are needed for imaging. The Combing-Imaging by Secondary Ion Mass Spectrometry (SIMS) (CIS) method helps solve these problems by combining DNA combing, cesium flooding, and quantitative imaging via the NanoSIMS 50. We show here that CIS can reveal, on the 50 nm scale, individual DNA fibers labeled with different, nonradioactive isotopes and, moreover, that it can quantify these isotopes so as to detect and measure the length of one or more short nucleic acid fragments associated with a longer fiber.



Much of our knowledge of DNA replication comes from studies of heterogeneous populations of DNA molecules. To gain insight at the level of the single molecule, Bensimon and co-workers developed the DNA combing method, which combines labeling DNA (e.g., via incorporation of modified nucleotides), combing this DNA on silanized glass, and direct and/or indirect detection of labeled DNA by fluorescence microscopy.[1−3] This method allows the observation of a large number of individual DNA fibers, several tens of kilobases long, lying parallel to one another. DNA combing has been used to study DNA metabolism in organisms with large genomes,[4] to investigate initiation[5] and elongation[6,7] of replication in eukaryotic cells and to map genes[4,8] or rRNA-coding regions.[9] It has also been used to study DNA rearrangements.[10,11]

The DNA combing method, which depends on fluorescence microscopy, suffers from several limitations: (i) incorporation of modified nucleotides may perturb replication, (ii) antibodies may not always recognize labeled DNA, (iii) the number of types of molecules that can be studied is usually limited to two or three, and (iv) resolution is limited to that of the optical microscope. Alternative methods to combing on silanized glass have been developed. These include combing on coverslips coated with polyelectrolytes[3] and DNA stretching in microchannels under capillary tension.[12] However, these techniques, even with quantum-dot end-labeling,[13] also have the limitations associated with fluorescence microscopy.

To overcome these limitations, we have developed a new method that combines DNA combing with the exquisitely sensitive imaging technique of dynamic secondary ion mass spectrometry (D-SIMS): the combing-imaging by secondary ion mass spectrometry (SIMS) method (CIS). Our objective in developing this method is to obtain fine-scale, quantitative information on DNA replication and protein—DNA interactions at the level of single molecules. The principle of SIMS is shown in Figure 1. The surface of a sample is rastered with a primary, highly energetic ion beam (here Cs^+); this primary beam fragments the sample molecules. In the analysis of many organic systems using a SIMS machine in the static regime with a primary ion fluence well below 10^{13} ions cm^{-2}, it is likely that the majority species of these cluster secondary ion fragments are either damaged molecular groups or simple fragments of the original molecule. In our analysis, using a NanoSIMS 50 in the dynamic

Received: June 30, 2011
Accepted: August 18, 2011
Published: August 18, 2011

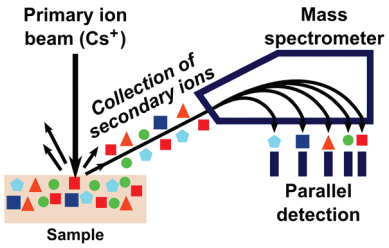


Figure 1. Principle of dynamic secondary ion mass spectrometry (D-SIMS).

regime with a primary ion fluence well above 10^{13} ions cm^{-2} (here 5×10^{14} ions cm^{-2}), the primary beam fragments the sample molecules down to monatomic ions that are then sputtered out.

Ions from within a few nanometers of one another can recombine to generate small charged molecular ions (e.g., CN^- species in the case of nitrogen containing organic samples).[14,15] The sputtered ions are collected (secondary beam) and analyzed in a mass spectrometer. The CAMECA NanoSIMS 50 can be used to detect and quantify ions under 300 Da with a very high mass resolution at high transmission to yield an isotopic image of the surface with a lateral resolution of around 50 nm.[16,17] With the NanoSIMS 50, the proportion of ions recovered (the useful yield) from the sample is close to or even greater than 3% of the CN^- ions produced under bombardment of C- and N-containing samples.[18] Hence, a combed DNA with a region 50 nm long containing 150 bp (or around 1000 nitrogen atoms) could yield a signal of around 30 CN^-. The NanoSIMS 50 allows a sample to be imaged at 5 different masses simultaneously and can discriminate between isotopes of the same element; hence, it can distinguish between $^{12}C^{14}N^-$, $^{13}C^{15}N^-$ and either $^{12}C^{15}N^-$ or $^{13}C^{14}N^-$.[19] It should be noted that forming the recombinant $^{13}C^{15}N^-$ ion in material containing elements with isotopes at their natural levels is 25 000-fold less likely than of forming the $^{12}C^{14}N^-$ ion. In principle, therefore, the discriminatory capacity of SIMS, combined with its sensitivity and spatial resolution, could be used to identify and quantify stretches of DNA labeled with various densities of different isotopes.

In the NanoSIMS 50 in D-SIMS, a normal incidence beam of Cs^+ primary ions is focused, down to 50 nm, on a point of the surface of a sample. The ionized portion of the particles sputtered by this primary beam is collected during a given time (dwell time, here typically 10 ms). These particles (secondary ions) are collected and then mass-filtered by a magnetic sector mass analyzer. Five masses are simultaneously counted by detectors (electron multipliers). By scanning a chosen area on the sample with the primary beam, five images (one per mass) originating from the exact same sputtered volume are simultaneously acquired. The first part of the secondary beam, coaxial with the primary beam, is not represented here.

Here, we describe the CIS method we have developed to analyze DNA combed on modified, SIMS-compatible, silicon surfaces. We show that isotopically labeled, single DNA fibers uniformly aligned on this surface can be detected with a resolution of 50 nm (i.e., 150 bp) using the NanoSIMS 50.

MATERIALS AND METHODS

Strains and DNA Preparation. The 168 (trpC2) strain of *Bacillus subtilis* and a derivative (MT119, trpC2 leuB6 r- m-) harboring a 21.1 kb long, chloramphenicol-resistant plasmid (pHV1431 plus insert of 10.3 kb)[20] were used in this work to extract chromosomal and plasmid DNA, respectively. Unlabeled DNA was prepared from cells grown in a rich medium (LB) supplemented or not with chloramphenicol 6 μg/mL. To prepare isotopically labeled DNA, cells were grown ~20 generations in minimal medium (14 g/L K_2HPO_4, 6 g/L KH_2PO_4, 1 g/L sodium citrate, 10 mM $MgSO_4$, 0.005% Trp, 0.005% Leu) supplemented with 0.2% ^{13}C-glucose (99% ^{13}C Isotec), 600 mM of $^{15}NH_4Cl$ (98% ^{15}N Isotec), and chloramphenicol when required. The following method was used to prepare chromosomal DNA free of peptides and RNA species. A volume of 2 mL of a freshly saturated culture were centrifuged and the pelleted cells were resuspended in 0.5 mL of lysis buffer (TrisHCl pH 8, 50 mM; EDTA, pH 8, 10 mM; NaCl, 150 mM; and lysozyme 5 mg/mL). After 20 min at 37 °C, samples were treated with proteinase K (0.2 mg/mL) and sarcosyl buffer (1.2%) for 20 min at 65 °C. Peptides and cell fragments were removed by a phenol/chloroform treatment. The aqueous, nucleic acid containing phase was then incubated with RNase (0.2 mg/mL, 10 min at 37 °C) and purified by a second phenol/chloroform extraction. The DNA was recovered by precipitation with 100% cold ethanol and centrifugation at 13 000*g* for 20 min at 4 °C in a benchtop centrifuge. The precipitated DNA was washed in 70% cold ethanol, centrifuged 10 min at 4 °C (13 000*g*), and dried 10 min under vacuum. The dried DNA was carefully resuspended in water for at least 12 h at room temperature. Finally, the DNA concentration, measured by absorbance at 260 nm, was adjusted to 0.2−2 μg/mL by dilution in NaCl or CsCl in a range of concentrations from 0 to 1 M. Plasmid DNA was extracted using the PureYield plasmid midiprep system (Promega Corporation, Madison, WI). Before CIS was performed, the plasmid DNA was linearized by *Pst*I according to suppliers' instructions (New England BioLabs, Inc., Hitchin, U.K.), purified by the phenol/chloroform procedure, and recovered by ethanol precipitation in the presence of 0.3 M potassium acetate.

To evaluate the effect of molecule size on combing, DNA fragments of various sizes were generated by PCR using as a template the genomic DNA of a *Bacillus subtilis* strain (HVS624).[21] PCR products of 2.94−4.9 kb were generated using the TaKaRa Ex Taq system as recommended by the supplier (Takara Shuzo Co., Ltd., Shiga, Japan). Products of 8.96 and 10.13 kb were generated using the Expand Long Template PCR System from Roche Applied Science (Mannheim, Germany). The fragments start from a single position in the bacterial genome (primer BD251, ACCAATGCTTAATCAGTGAGGC) and end at various positions (primers LJ145, CGGAATTCACTGACGAGT TCTTCCACAGC (2937 bp); ELC161, TCACCTTTGGAACACTTGC (4095 bp); ELC162, GATAATCTGTGCGACGTGC (4942 bp); JC200, AGACGCGCGATTTGAACCCGG (8961 pb); JC195, GCTTAGTCCCTCCACGATGTA (10 131 pb)). The PCR products were purified using the QIAquick PCR Purification kit (QIAGEN GmbH, Hilden, Germany). The plasmid DNA and PCR products were diluted for CIS as described above for genomic DNA.

Preparation of Silicon Surfaces. *Preparation of Hydrogenated-Terminated Silicon Surfaces (Si−H).* The silicon wafers used in this study were p-type, boron-doped, Si (100) (ρ > 1 Ω cm) (Siltronix, Archamps, France). The silicon substrate was cleaned in an ultrasonic bath for 5 min periods in acetone and isopropylic alcohol followed by extensive rinsing with ultrapure water. The substrate was then immersed for 20 min in a "piranha" solution (H_2SO_4/H_2O_2 3/1) to remove organic contaminants

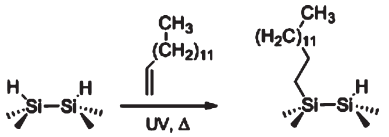


Figure 2. Schematic illustration of the chemical functionalization of hydrogen-terminated silicon surfaces via a hydrosilylation reaction using 1-tetradecene.

on the surface. The clean surface was immersed in a dilute aqueous solution of HF (50%) to generate a hydrogen-terminated surface (Si—H),[22] then extensively washed with water, and blow-dried with nitrogen. The resulting surface had a contact angle of 85° for a 1 μL water droplet. Freshly prepared Si—H surfaces were used immediately for DNA combing or for grafting hydrocarbon chains (see below).

Formation of Organic Monolayers on Hydrogen-Terminated Silicon Surfaces. The hydrosilylation reaction of 1-alkene with the hydrogen-terminated silicon surface results in the formation of an organic monolayer covalently linked through stable Si—C bonds (Figure 2).[22] Hence, this reaction was used to obtain highly hydrophobic surfaces by grafting alkenes with different alkyl chains (C_6, C_{10}, and C_{14}) onto freshly prepared Si—H surfaces. The freshly hydrogen-terminated silicon surfaces were placed under nitrogen in a Schlenk tube containing deoxygenated neat 1-alkene and irradiated at 312 nm in a photochemical reactor for 3 h. The excess of unreacted and physisorbed reagent was removed by rinsing, at room temperature, with chloroform and ethanol, and the sample was dried under a stream of nitrogen. Water contact angles were measured using deionized water. All measurements were made in ambient atmosphere at room temperature. A remote, computer-controlled, goniometer system (DIGIDROP by GBX, France) was used to measure the contact angles to an accuracy of ±2°.

Methods for DNA Combing on Silicon. The "drop"and "lift" methods[1,4] were both used to comb DNA on the Si—C_{14} surface while only the drop method was used for Si—H surfaces. Both the "drop" and "lift" methods entail the DNA becoming attached to the surface and then drawn out and aligned perpendicular to the triple line or the meniscus, respectively. In the case of the "lift" method, part of the silicon wafer was immersed in a DNA solution. The wafer was then mechanically pulled out of the solution at a constant speed (600 μm/min) and rapidly air-dried. In the "drop" method, 10 μL of a DNA solution was deposited on the surface. After 10 min of incubation, the wafer was (i) tilted with tweezers to 45° to cause the drop to roll off the surface, (ii) washed by immersion in water, and (iii) rapidly air-dried. It should be stressed here that coated silicon wafers must be protected from dust and thoroughly dried before combing because a thin film of water strongly impedes DNA adsorption. Surfaces should also be washed with water after combing to avoid perturbation of D-SIMS analysis by saline crystals. We checked using AFM that washing does not desorb combed DNA.

D-SIMS Methods. *Au Method.* In D-SIMS, implantation of Cs^+ ions of the primary beam in the surface is required for an efficient ionization of the atoms sputtered from the sample. It is thus difficult to observe thin biological samples (like single molecules) with this technology because they are eroded by the primary beam before sufficient Cs implantation and subsequent efficient ionization. One way to avoid this problem is to coat samples with Au. We therefore deposited (using a Cressington sputter coater) a layer of Au, approximately 60 nm thick, on wafers with combed DNA. Even though we succeeded in obtaining images of combed DNA with Au coating, their quality was poor (not shown).

Cs Flooding Method. The deposition of neutral Cs on samples combined with bombardment with a beam of primary Cs^+ ions has advantages over bombardment without deposition:[23,24] (i) the Cs surface concentration can be varied so as to maximize useful yields of secondary ions and (ii) the time to reach the steady state (pre-equilibrium regime) can be decreased thereby avoiding destruction of thin samples before analysis. Therefore, we developed a system to deposit neutral cesium on samples (the cesium flooding system). It comprises a neutral cesium evaporator and an independent stand-alone UHV chamber. A vacuum suitcase was made to transport samples and to transfer them to different analytical instruments under ultrahigh vacuum conditions (10^{-8} to 10^{-10} Torr). This avoids an immediate reaction between the neutral cesium deposit and air. To analyze combed DNA, the wafer surface was flooded with Cs^0 at 1.5 Å/s for 1800 s before imaging with the CAMECA NanoSIMS 50.

NanoSIMS 50 Analyses. Wafers with combed DNA were analyzed in the multicollection image mode of the NanoSIMS 50 ion analyzers (Cameca, Gennevilliers, France) in both Rouen and Belvaux. Wafers that had undergone cesium flooding were analyzed in Belvaux. Briefly, the NanoSIMS 50 was used in the negative secondary-ion mode with the Cs^+ primary ion beam. High spatial resolution images of 256 × 256 or 128 × 128 pixels were obtained using a primary beam around 1 pA in intensity, the D1-2 or D1-3 diaphragms, and dwell times as indicated in the figures. Secondary ions were energy-filtered in order to obtain a minimal mass resolution of 5000 (10% height peak measurement). The images of the ^{12}C and/or ^{13}C, $^{12}C^{14}N$, $^{12}C^{15}N$ or $^{13}C^{14}N$, $^{13}C^{15}N$ and sometimes ^{32}S distributions were simultaneously acquired.

Safety Considerations. The piranha solution is a strong oxidant. It reacts violently with organic materials. It can cause severe skin burns. It must be handled with extreme care in a well-ventilated fume hood while wearing appropriate chemical safety protection. HF is a hazardous acid that can cause serious tissue damage if burns are not appropriately treated. Etching of silicon should be performed in a well-ventilated fume hood with appropriate safety considerations: face shield and double-layered nitrile gloves.

RESULTS

Modifying the Surface of Silicon Wafers for CIS. Silicon wafers are known to be compatible with SIMS because of their purity, flatness, and conductivity. In developing the CIS technique, we needed to prepare silicon wafers with hydrophobic surfaces to permit DNA combing. To achieve this, we first tested a Si—H surface that had the advantage of not containing C and N atoms and therefore of not generating a CN background (although a low background might still be generated by contaminants containing C and N). Such a low background is important for SIMS (see below). Unfortunately, this Si—H surface was of moderate hydrophobicity (the water contact angle was 85°) and atomic force microscopy (AFM) studies showed that the DNA combing was of poor quality (not shown). We thus tested wafers grafted with C_6, C_{10}, and C_{14} alkenes and found that the hydrophobicity of their surfaces was substantially higher than that of the Si—H surface (the water contact angle was 101° for the

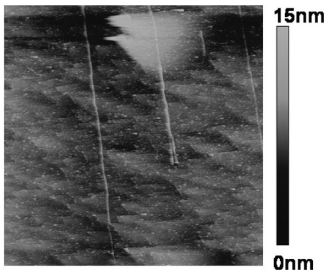


Figure 3. Example of an AFM image of combed DNA on a Si−C_{14} wafer. Chromosomal *Bacillus subtilis* DNA 0.2 μg/mL combed on silicon wafer grafted using the hydrosilylation reaction. Field of view 6 μm × 6 μm; tapping mode. The recorded heights are between 0 and 15 nm (see scale). The fibers were combed from bottom to top (i.e., the direction of runoff of the solution).

Si−C_6 surface and of 104° for the C_{10}- and C_{14}-terminated surfaces). Moreover, AFM studies (Figure 3) showed that combing was also improved on these surfaces, particularly on the Si−C_{14} surface. Therefore most of the following experiments were carried out on Si−C_{14} surfaces.

Combing Methods. Two methods were used to comb DNA. They gave similar results although combing is more reproducible and of better quality with the tightly controlled, motor-driven "lift" method than with the manual "drop" method. That said, the lift method does need more DNA and takes longer.

We also analyzed the influence of DNA size on combing using PCR products (2.94, 4.09, 4.94, 8.96, and 10.1 kb). AFM imaging showed that the "drop" method was satisfactory with fragments equal to or greater than 4.94 kb while SIMS imaging showed that the lift method was better than the drop method for molecules smaller than this.

Influence of DNA Concentration and Ionic Composition on Combing. The quality of combing on glass depends to a large extent on the concentration of DNA, a relationship that has been shown to exist on mica.[25] On modified silicon surfaces and at high DNA concentrations (>1 μg/mL), both SIMS and AFM imaging showed highly branched, "rope-like" DNA structures. This was observed whatever the nature of DNA (chromosome, plasmid, phage, or PCR product) and size (from 10 to 100 kb). At lower concentrations (~0.2 μg/mL), most of the DNA is combed as single fibers (Figure 3). Still lower concentrations resulted in satisfactory combing but with an insufficient number of readily visible fibers on the images. Hence, the DNA concentration we chose for combing was 0.2 μg/mL although this concentration was slightly modified according to the average size so as to obtain the desired density of combed molecules.[4] It should be noted that incomplete solubilization of ethanol-precipitated DNA severely perturbs combing.

Finally, because Na^+ and Cs^+ ions have a different effect on water structure, we tested combing in the presence of different ionic strengths (from 0 to 1 M) of Na^+ and Cs^+ chloride. Media containing NaCl gave better combing than those containing CsCl, and the best combing was obtained with ionic strengths around 0.2 M.

Single Molecule Analysis in Dynamic SIMS. In the dynamic SIMS technique with a Cs^+ primary ion beam, a process of Cs^+ implantation is needed before reaching a steady regime of secondary ion emission. In this process, the increasing concentration of Cs trapped in the bombarded surface results in an increasing probability of forming negative ions. As a result of this characteristic, the basic D-SIMS methodology cannot allow detection of thin samples like DNA fibers on a surface because this material is sputtered out before the steady state regime is reached that would allow its detection. This constituted a serious problem. One solution would be to coat the surface to allow Cs^+ implantation to occur before the primary beam erodes the surface down to the DNA. We therefore coated the combed DNA with a 60 nm thick layer of Au, but even though we succeeded in obtaining images of combed DNA with Au-coated surfaces, their quality was low.

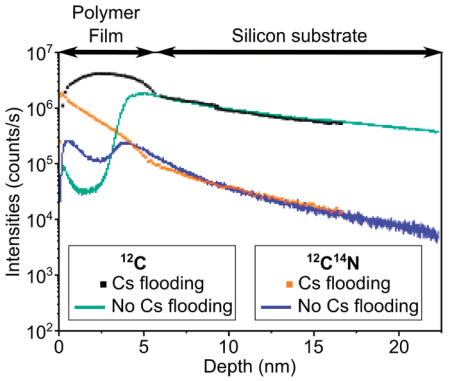


Figure 4. Value of cesium flooding in D-SIMS technology. Intensities of ^{12}C and $^{12}C^{14}N$ from NanoSIMS 50 analysis of a 5 nm thick polyvinyl-4-pyridine film, with (continuous line) and without (dotted line) cesium flooding.

We have previously shown that quantitative measurements can be obtained when the cesium concentration in a surface is greater than a threshold.[26,27] We therefore tested the effect of Cs coating on imaging of combed DNA. For this, we developed a system, termed thereafter "cesium flooding", to deposit neutral Cs on surfaces. In contrast to Au coating, Cs flooding gave high quality images (see below). This process was thus used in the following experiments.

To investigate the value of cesium flooding for D-SIMS analysis of biomolecules and, in particular, its value to CIS, we used information acquired from analysis of a 5 nm thick poly-(vinyl-4 pyridine) film on the surface of a silicon wafer; this film was analyzed under Cs^+ bombardment using a NanoSIMS 50 with and without neutral cesium deposition (Figure 4). A dramatic decrease in the duration of the pre-equilibrium regime (the time to reach the steady state) was observed for both $^{12}C^-$ and $^{12}C^{14}N^-$ secondary ions and, hence, in the corresponding depth of material sputtered out before reliable recovery of material. From the relationship between the intensities and the depth sputtered, we calculated that, for example, the useful yield of carbon C^- was increased from 7.1×10^{-3} with the standard technique of Cs^+ ion bombardment alone to 1.2×10^{-1} when neutral cesium flooding was used. This result shows that cesium flooding is useful (and in some cases indispensable) for imaging biomolecules such as DNA and proteins[28] on solid surfaces.

CIS Allows High-Resolution Imaging of Single DNA Fibers. To demonstrate that CIS can reveal combed DNA, isotopically labeled *B. subtilis* chromosomal DNA was substantially enriched in vivo with ^{13}C and ^{15}N. The extracted DNA was then combed on Si−C_{14} using the "drop" method, washed with water, covered with neutral cesium, and imaged using a NanoSIMS 50. Images of the same field at various masses were simultaneously collected as illustrated (Figure 5).

dx.doi.org/10.1021/ac201685t |*Anal. Chem.* 2011, 83, 6940–6947

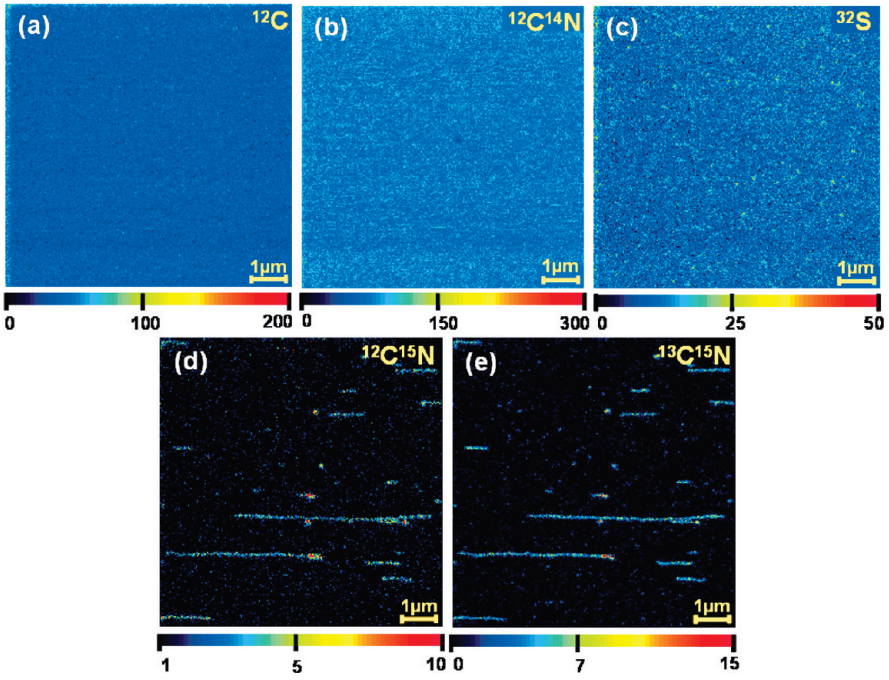


Figure 5. SIMS images of double-labeled (^{13}C, ^{15}N) chromosomal *Bacillus subtilis* DNA. The DNA was combed at 0.2 μg/mL on a Si−C_{14} wafer, the wafer surface was covered with cesium and analyzed with a NanoSIMS 50. Primary beam intensity, 1 pA; dwell time, 10 ms; field of view, 8 μm × 8 μm; 256 × 256 pixels; bars = 1 μm. Acquired masses are (a) ^{12}C, (b) $^{12}C^{14}N$, (c) ^{32}S, (d) $^{12}C^{15}N$, and (e) $^{13}C^{15}N$. The count numbers are given on the color scale beneath each image. For image d, pixels at 0 and 1 count are displayed in black. The DNA was combed from left to right (the combing direction is coincidentally parallel to the abscissa).

The homogeneous distribution of ^{12}C (Figure 5a) indicates that the silicon surface was uniformly grafted with the C_{14} alkyl chains and uniformly sputtered by the primary Cs^+ beam. A homogeneous and rather high 110 ± 12 counts per pixel (mean ± standard error) distribution of $^{12}C^{14}N$ was also observed in the analyzed fields (Figure 5b). This indicates that the surface contains nitrogen even though nitrogen-containing molecules were not used during the preparation of either the wafer surfaces or the DNA (this DNA was dissolved in water or in 0.2 M NaCl rather than in solutions containing organic, aminated compounds like TRIS). Given that (i) combed DNA contains ^{15}N rather than ^{14}N and that (ii) the $^{12}C^{15}N/^{12}C^{14}N$ ratio in areas lacking DNA was close to that expected for natural samples (the measured ratio was 0.40% while the natural ratio is 0.36%), the ^{14}N detected on the wafer surface probably originates from contaminants. ^{32}S contamination was also detected (Figure 5c). Thus contamination, even in trace amounts, is an important problem for the highly sensitive CIS method. Developing a better protection of samples from contamination is essential to fully exploiting the quantitative capacity of the D-SIMS technology.

Nitrogen atoms readily recombine with carbon atoms during sputtering to generate CN^- ions; this occurs with a probability that decreases strongly when the initial distance separating the atoms under the beam is more than around 2 nm.[15] The DNA fibers were strongly enriched in ^{13}C and ^{15}N, and $^{12}C^{15}N$ and $^{13}C^{15}N$ masses were therefore collected. The distributions of these masses were essentially identical. They revealed parallel linear fibers of various lengths (Figure 5d,e). The CN^- values of these fibers (14 ± 4 counts per pixel for $^{12}C^{15}N$ and 17 ± 5 counts per pixel for $^{13}C^{15}N$) are highly significant, albeit low. In the absence of labeling, we should have obtained signals of only 4 and 0.4 counts per pixel for $^{12}C^{15}N$ and $^{13}C^{15}N$, respectively, given the $^{12}C^{14}N$ value of the surface (110 counts per pixel) and the natural isotopic abundances for ^{13}C and ^{15}N. This demonstrates that the detected material originates from double-labeling, and since the combed material was treated with an RNase and a protease, it corresponds to DNA and not to RNA or protein.

Similar amounts of $^{12}C^{15}N$ and $^{13}C^{15}N$ (Figures 5d,e) are formed because the atomic recombination that occurs during sputtering results in ^{15}N in the DNA recombining with C ions from either the DNA (^{13}C) or from the alkyl-chains (^{12}C). Note again that this recombination occurs within only a few nanometers.

The quantitative information provided by CIS allowed us to study the variation in total ^{15}N counts (defined as ($^{13}C^{15}N$ + $^{12}C^{15}N$)) along the DNA fibers. Several of the fibers in Figure 6 show an increased number of counts at the extremity that was combed last (the right end of the fibers). It is conceivable that the increased counts at the end of the fibers are due to the difficulty of untangling the last stretch of DNA before it is attached to the surface. In some cases, however, the increase in counts occurs elsewhere in the fiber. We therefore analyzed such a region (white rectangle in Figure 6a,b) by scanning it using a vertical window 5 pixels high and 1 pixel wide in which the counts of the 5 pixels within the window were added to one another. $^{13}C^{15}N$, $^{12}C^{15}N$, and total ^{15}N counts are shown in Figure 6c. Within the region, there are four sections that can be distinguished because they have differences in average counts that are clearly greater than the fluctuations within these sections. In section B, the total ^{15}N count per pixel is 31 ± 6; this value allows the useful yield to be estimated at 0.02 which is consistent with expectations (see above). This would be consistent with the fiber in section B corresponding to a single DNA molecule. AFM studies support this interpretation since they showed that most of the fibers detected upon combing DNA on grafted silicon surfaces at low

dx.doi.org/10.1021/ac201685t |*Anal. Chem.* 2011, 83, 6940–6947

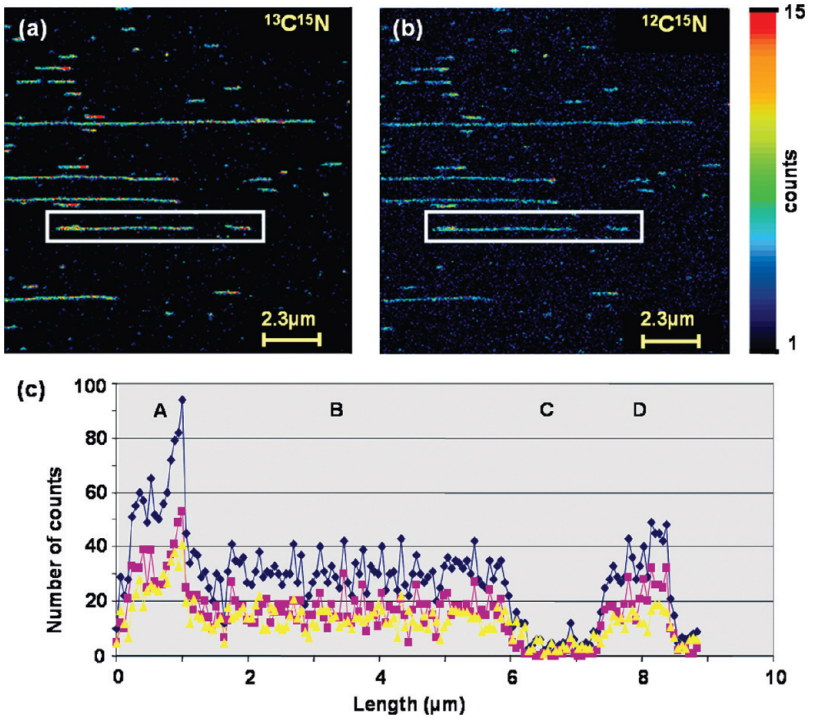


Figure 6. CIS-based analysis of ^{15}N content of a DNA fiber. (a and b) SIMS images of double-labeled (^{13}C, ^{15}N) chromosomal *Bacillus subtilis* DNA combed at 0.2 μg/mL on a Si−C_{14} wafer. The wafer surface was covered with cesium and analyzed with a NanoSIMS 50. Primary beam intensity, 1 pA; dwell time, 10 ms; field of view, 15 μm × 15 μm; 256 × 256 pixels; bars = 2.3 μm. Acquired masses are (a) $^{13}C^{15}N$ and (b) $^{12}C^{15}N$. The count numbers (1−15) are given on the color scale on the right. (c) Variation of the count number along the fiber is shown in the white rectangular window in parts a and b. Each curve is the total number of counts calculated from 5 lines of pixels in the window (pink squares, $^{13}C^{15}N$; yellow triangles, $^{12}C^{15}N$; blue diamonds, total count numbers $^{13}C^{15}N$ + $^{12}C^{15}N$). The DNA was combed from left to right.

Table 1. Diameter of DNA Fiber Estimated Using AFM by Measuring the Height of 49 Fibers

fiber height (nm)	<1	1−2	2−3	3−4	4−5	5−6	6−7	7−8
number of fibers	8	30	5	1	0	3	1	1

concentration (0.2 μg/mL) are individual molecules (Figure 3). In section A, the total ^{15}N count is more than 60, which probably corresponds to two or more individual fibers (or to a fiber that has doubled back on itself). Section C has a background level of counts and corresponds to a gap between separate fibers. Section D reveals a higher level of counts at the right extremity, which is probably indicative of the difficulty of finishing combing the fiber leading to a locally greater density of DNA. Similar results were obtained for 87 DNA fibers in six separate images.

AFM measurement confirmed that most of the fibers were single fibers since the heights of the chromosomal DNA fibers combed on Si−C_{14} wafers (Table 1) were close to (i) the diameter of single noncombed DNA fibers measured by AFM by others[29] and (ii) the known diameter of DNA in the B conformation. Taken together, these results show that CIS allows high-resolution imaging of single DNA fibers.

CIS and DNA Conformation. ^{13}C- and ^{15}N-labeled 21.1 kb plasmids, cut at a single position, were combed on Si−C_{14} wafers. Figure 7 is an example of SIMS images. The average length of a set of 21 molecules measured on SIMS images was 4.4 ± 0.5 μm. This value is shorter than the 5.46−6.09 μm and the 7.14 μm expected for DNA in the A and B conformations, respectively. Clearly, the physicochemical environment of the

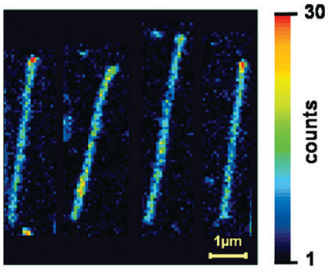


Figure 7. SIMS images of 4 double-labeled (^{13}C, ^{15}N) linearized plasmids of *Bacillus subtilis* (21.1 kb). The plasmids were combed on a Si−C_{14} wafer. The wafer surface was covered with cesium and analyzed with a NanoSIMS 50. Primary beam intensity, 1 pA; dwell time, 10 ms; 256 × 256 pixels. The figure is an assembly of 4 plasmid images for $^{13}C^{15}N$ secondary ion (bar = 1 μm). The count numbers (1−30) are given on the color scale. The DNA was combed from bottom to top.

DNA on the surface and the preparation of the DNA are important.[24] One interpretation is that the DNA fibers are anchored to the surface at some points and "float" between these points. This would mean that the DNA between two anchorage points is actually longer than the distance between these points. This interpretation of imperfect adherence of DNA between points of stronger anchorage is supported by (i) the ease with which the DNA fibers wrap around the AFM tip and (ii) displacements of short segments of DNA under the Cs^+ beam during the SIMS imaging.

On Si−H surfaces, the lengths of combed 10.13 kb PCR fragments measured from SIMS images were about 2.9 μm

(not shown), which can be compared with the 2.63−2.94 μm expected for the A form and the 3.44 μm expected for the B form. The difference between these values in terms of nanometers/base pair and those measured when the combing is carried out on Si−C_{14} wafers is likely due to the lower hydrophobicity of the Si−H surface. This may be compared with DNA combed on glass where stretching gives lengths 110−150% of the original in the B conformation.[1−3] It is thus likely that CIS yields a DNA with conformations different from those of DNA combed on glass.

DISCUSSION

Studies of DNA replication, repair, and recombination often require techniques that provide high sensitivity and high resolution and that do not perturb the processes under study. To respond to this need, we have developed CIS to exploit the combined advantages of DNA combing and SIMS. This development entailed combing DNA fibers on modified silicon surfaces, flooding these surfaces with cesium, and then using a NanoSIMS 50 to image on the 50 nm scale sets of individual DNA molecules.

DNA fragments as short as 1500 nm were combed successfully and were imaged at a resolution of 50 nm. This allowed such a fragment to be characterized as a sequence of 30 regions (1500/50). This is a substantial improvement over fluorescence imaging in which a 1500 nm fragment could be characterized as 2 or 3 regions. These minimal regions correspond to around 200 bp in CIS and 2000 bp in fluorescence. This improved resolution holds for longer DNA fragments of up to 20 μm with the NanoSIMS. Fragments of chromosomal DNA as short as 200 nm can also be seen when the lift method was used (Figures 5 and 6), possibly because this gives better combing than the drop method.

DNA fibers can form duplexes or even multiplexes[25,30] which, if combed, must be detected. Such detection by fluorescence can be difficult due to problems with sensitivity and quantification. Detection with CIS is relatively easy since, for example, $^{13}C^{15}N$ counts are proportional to the number of associated DNA fibers (compare DNA sections A and D with section B in Figure 6c).

Labeling with the nonradioactive isotopes of the constitutive molecules of cells, such as the ^{13}C and ^{15}N that we have used here, is generally known to not significantly perturb cells. Labeling is not limited to these isotopes and, if necessary, other isotopes could be used in CIS. These include modified nucleotides containing Br or I atoms (or other brominated or iodinated molecules), which can be detected with high sensitivity using SIMS.

In principle, CIS could be used to identify origins of replication or to study local variations in the rate of DNA elongation resulting from signals generated inside or outside cells or indeed from addition of drugs. This would entail using pulse-labeling to incorporate isotopically distinguishable nucleotides into short lengths of DNA and would allow subsequent CIS to image adjacent regions of DNA containing isotopes in different ratios even when these regions are only a couple of hundred base pairs long (see above). CIS could, for example, permit comparisons of velocities of replication measured after incorporation in yeast of deoxybromouridine (which can perturb this velocity)[5] or ^{13}C-/^{15}N-labeled thymidine.

CIS could be used to study the interaction between DNA sequences and proteins, polyamines, and other molecules, including drugs. This could be achieved by differential isotopic labeling of DNA and proteins or other molecules. Hence, further development of CIS may yield unique insights into the composition and location of DNA-binding complexes.

CONCLUSIONS

A new technique, CIS, has been developed to allow the isotopic analysis of individual DNA fibers based on combing DNA fibers on modified silicon surfaces, flooding these surfaces with cesium, and imaging via SIMS. DNA fragments of 1500 nm (or 4500 bp) were imaged at a resolution of 50 nm (or 150 bp). Using a NanoSIMS 50 to image at 5 different masses simultaneously, we showed that CIS can discriminate between unlabeled DNA and DNA labeled with ^{13}C and ^{15}N. Such nonradioactive isotopes are not believed to perturb cells. CIS could help reveal the timing of initiation at different origins of replication and variations in the rate of DNA elongation. Moreover, the technique could be extended to studying the interaction between DNA and other molecules, including proteins and drugs. CIS should therefore constitute a precious tool for the study of processes such as DNA replication, recombination, and repair.

AUTHOR INFORMATION

Corresponding Author

*E-mail: victor.norris@univ-rouen.fr.

ACKNOWLEDGMENT

This work was supported by the EU Network of Excellence, Nanobeams No. 500440.

REFERENCES

(1) Bensimon, A.; Simon, A.; Chiffaudel, A.; Croquette, V.; Heslot, F.; Bensimon, D. *Science* **1994**, *265*, 2096–2098.

(2) Bensimon, D.; Simon, A. J.; Croquette, V.; Bensimon, A. *Phys. Rev. Lett.* **1995**, *74*, 4754–4757.

(3) Chan, T. F.; Ha, C.; Phong, A.; Cai, D.; Wan, E.; Leung, L.; Kwok, P. Y.; Xiao, M. *Nucleic Acids Res.* **2006**, *34*, e113.

(4) Michalet, X.; Ekong, R.; Fougerousse, F.; Rousseaux, S.; Schurra, C.; Hornigold, N.; van Slegtenhorst, M.; Wolfe, J.; Povey, S.; Beckmann, J. S.; Bensimon, A. *Science* **1997**, *277*, 1518–1523.

(5) Lengronne, A.; Pasero, P.; Bensimon, A.; Schwob, E. *Nucleic Acids Res.* **2001**, *29*, 1433–1442.

(6) Herrick, J.; Stanislawski, P.; Hyrien, O.; Bensimon, A. *J. Mol. Biol.* **2000**, *300*, 1133–1142.

(7) Lebofsky, R.; Bensimon, A. *Mol. Cell. Biol.* **2005**, *25*, 6789–6797.

(8) Lebofsky, R.; Bensimon, A. *Briefings Funct. Genomics Proteomics* **2003**, *1*, 385–396.

(9) Caburet, S.; Conti, C.; Schurra, C.; Lebofsky, R.; Edelstein, S. J.; Bensimon, A. *Genome Res.* **2005**, *15*, 1079–1085.

(10) Gad, M.; Mizutani, W.; Machida, M.; Ishikawa, M. *Nucleic Acids Symp. Ser.* **2000**, 215–216.

(11) Gad, M.; Machida, M.; Mizutani, W.; Ishikawa, M. *J. Biomol. Struct. Dyn.* **2001**, *19*, 471–477.

(12) Sidorova, J. M.; Li, N.; Schwartz, D. C.; Folch, A.; Monnat, R. J., Jr. *Nat. Protoc.* **2009**, *4*, 849–861.

(13) Crut, A.; Geron-Landre, B.; Bonnet, I.; Bonneau, S.; Desbiolles, P.; Escude, C. *Nucleic Acids Res.* **2005**, *33*, e98.

(14) Lechene, C. P.; Luyten, Y.; McMahon, G.; Distel, D. L. *Science* **2007**, *317*, 1563–1566.

(15) Legent, G.; Delaune, A.; Norris, V.; Delcorte, A.; Gibouin, D.; Lefebvre, F.; Misevic, G.; Thellier, M.; Ripoll, C. *J. Phys. Chem. B* **2008**, *112*, 5534–5546.

(16) Guerquin-Kern, J. L.; Wu, T. D.; Quintana, C.; Croisy, A. *Biochim. Biophys. Acta* **2005**, *1724*, 228–238.

(17) Boxer, S. G.; Kraft, M. L.; Weber, P. K. *Annu. Rev. Biophys.* **2009**, *38*, 53–74.

(18) Hillion, F.; Daigne, B.; Girard, F.; Slodzian, G.; Schuhmacher, M. *Second. Ion Mass Spectrom.* **1993**, *IX*, 254–257.

(19) McMahon, G.; Glassner, B. J.; Lechene, C. P. *Appl. Surf. Sci.* **2006**, *252*, 6895–6906.
(20) Janniere, L.; Bruand, C.; Ehrlich, S. D. *Gene* **1990**, *87*, 53–61.
(21) Dervyn, E.; Suski, C.; Daniel, R.; Bruand, C.; Chapuis, J.; Errington, J.; Janniere, L.; Ehrlich, S. D. *Science* **2001**, *294*, 1716–1719.
(22) Boukherroub, R.; Bensebaa, F.; Morin, S.; Wayner, D. D. M. *Langmuir* **1999**, *15*, 3831–3835.
(23) Wirtz, T.; Migeon, H.-N. *Appl. Surf. Sci.* **2004**, *231–232C*, 940–944.
(24) Wirtz, T.; Migeon, H.-N. *Surf. Sci.* **2004**, *557*, 57–72.
(25) Hansma, H. G.; Revenko, I.; Kim, K.; Laney, D. E. *Nucleic Acids Res.* **1996**, *24*, 713–720.
(26) Philipp, P.; Wirtz, T.; Migeon, H.-N.; Scherrer, H. *Int. J. Mass Spectrom.* **2006**, *253*, 71–78.
(27) Philipp, P.; Wirtz, T.; Migeon, H.-N.; Scherrer, H. *Appl. Surf. Sci.* **2006**, *252*, 7205–7207.
(28) Audinot, J.-N.; Cabin-Flaman, A.; Philipp, P.; Legent, G.; Wirtz, T.; Migeon, H.-N. *Surf. Interface Anal.* **2011**, *43*, 302–305.
(29) Pope, L. H.; Davies, M. C.; Laughton, C. A.; Roberts, C. J.; Tendler, S. J. B.; Williams, P. M. *Anal. Chim. Acta* **1999**, *400*, 27–32.
(30) Danilowicz, C.; Lee, C. H.; Kim, K.; Hatch, K.; Coljee, V. W.; Kleckner, N.; Prentiss, M. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 19824–19829.