

A simple and optimized method of producing silanized surfaces for FISH and replication mapping on combed DNA fibers

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Molecular combing of DNA is an extremely powerful DNA fiber–stretching technique that is often used in DNA replication and genome stability studies. Optimal DNA combing results mainly depend on the quality of the silanized surfaces onto which fibers are stretched. Here we describe an improved method of liquid-phase silanization using trimethoxy-octenylsilane/n-heptane as novel silane/solvent combination. Our simple method produces homogeneously modified coverslips in a reproducible manner, but does not require any sophisticated or expensive equipment in comparison to other known silanization protocols. However, DNA fibers were combed onto these coverslips with very good high-density alignment and stayed irreversibly bound onto the surfaces after various denaturing treatments, as required for different immunofluorescent detection of DNA with incorporated modified nucleotides or FISH.

INTRODUCTION

Molecular combing of DNA combined with immunofluorescent detection is one of the most efficient techniques for stretching and visualizing single DNA fibers. Since its initial description over 10 years ago (1,2), molecular combing has been used for drawing high-resolution physical maps of genomic regions (3,4) or for the detection of chromosomal rearrangements such as deletions (4) and amplifications (5). It is also widely used to study DNA replication and genome stability. Sites of DNA replication (known as replication eyes) can be directly visualized on combed DNA fibers after proper labeling via incorporation of modified nucleotides into nascent DNA. A number of parameters, such as replication origin density, fork speed, and inter-origin distances, can be obtained from the labeled combed

molecules. Studies of replication using DNA combing have been realized in various models, such as the *Xenopus* in vitro system (6–9), in mammalian cell lines (10–13), and in both budding and fission yeasts (14,15). Furthermore, in order to study replication at specific loci, immunofluorescent detection of both hybridized probes and replicated DNA has been successfully carried out on combed fibers (10,11,16). Recently, a number of studies on DNA repair, DNA damage, and intra-S checkpoints using molecular combing have been published (17–20).

Molecular combing is a simple and reproducible fiber stretching technique (1,2) (Figure 1A). A chemically modified glass coverslip is dipped into a buffered DNA solution. DNA fibers bind to the chemically modified, hydrophobic surface by one or both of their extremities in a pH-dependent manner. When the coverslip is pulled out with a

slow and constant speed ($v = 300 \mu\text{m s}^{-1}$) (4), the receding meniscus stretches the anchored DNA molecules onto the coverslip as it applies a constant perpendicular force on them. This rapid process results in irreversibly fixed DNA fibers and has the major advantage—in comparison to other known fiber-stretching techniques—that DNA fibers are aligned in parallel all over the surface. The stretching factor is constant ($1 \mu\text{m} \sim 2 \text{ kb}$) (1,2), so that internal size standards are not necessary once calculated under the same lab conditions.

Most of the current applications of molecular combing rely on good density and alignment of the combed molecules. Both depend on the quality of the coverslip surface modification, which has to be homogeneous to support DNA binding on its entire surface. Different surface modifications have been studied, but the chemical

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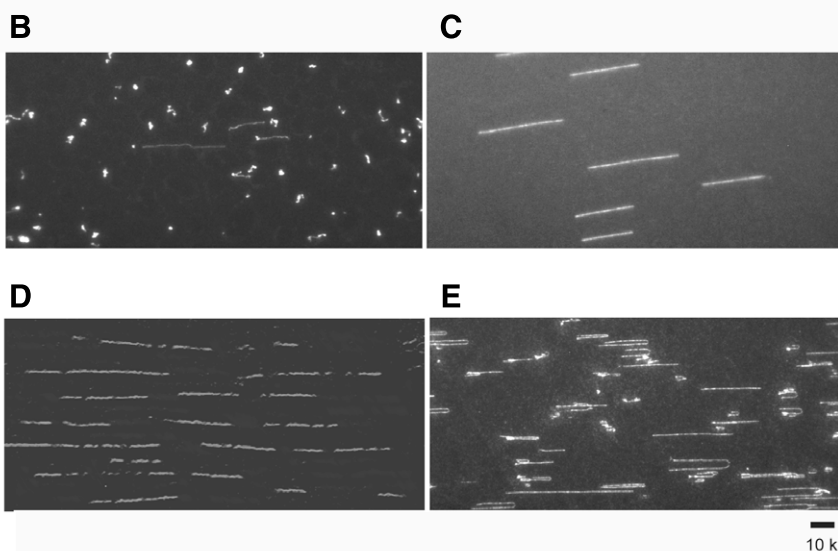
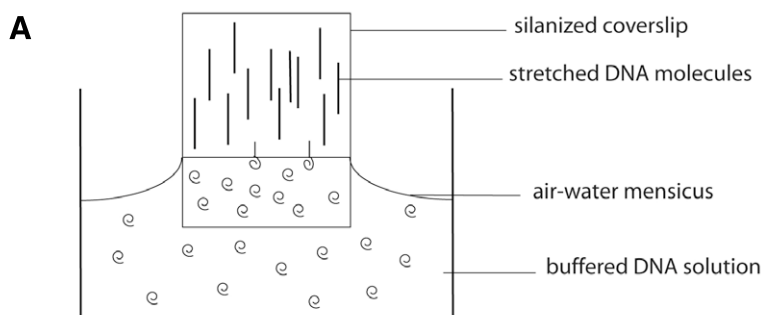


Figure 1. DNA combing on various surfaces. (A) Principle of DNA combing: DNA fibers are stretched on a silanized coverslip by a receding air-water meniscus (adapted from Reference 4). (B–E) λ phage DNA stained with YOYO-1 was combed onto different surfaces and immediately visualized by fluorescent microscopy. λ phage DNA was combed on (B) an untreated glass coverslip, (C) a polystyrene-coated surface, (D) a trimethoxy-octenylsilane coated surface, and (E) a trichloro-octenylsilane coated surface.

surface modification ensuring a best-possible stretching of the DNA fiber is the coating of the surface by an octenyl carbon chain (1,21), achieved by a silanization reaction (22). Only this surface modification assures that DNA fibers are irreversibly fixed and are not lost during treatments, especially from denaturation of combed double-stranded DNA by chemicals or heat, which is necessary for FISH and detection of replication eyes.

The key point for a wider utilization of the DNA combing technique is the availability of high-quality hydrophobic silanized coverslips, which, so far, are not commercially available and need to be homemade. Several silanization methods for surfaces exist, but the most adapted and reproducible coverslips for molecular combing have been obtained, until now, by gas-phase silanization

methods (1,21). However, gas-phase silanization requires controlled anhydrous conditions in specialized incubators, which are difficult to use in common biology laboratories. Here, we describe an improved and reproducible liquid-phase silanization method using a novel solvent/silane combination that is practicable in just about any laboratory. We further validate and characterize the silanized surfaces for molecular combing of DNA and its applications in molecular biology.

MATERIALS AND METHODS

Chemicals and products

All chemical products need to be of the highest quality. Acetone, methanol,

and chloroform were purchased from VWR France (Fontenay-sur-Bois, France). All other chemicals and (7-octen-1-yl) trimethoxysilane (Catalog no. 452815) were purchased from Sigma-Aldrich France (Saint Quentin Fallavier, France). We used 20 × 20 mm or 22 × 22 mm glass coverslips purchased from Esco (Portsmouth, VA, USA). Coverslips were generally silanized in a batch of no more than 25 units.

Combing apparatus

Our combing device was purchased from the Pasteur Institute (Paris, France), which no longer sells the machine commercially. Motorized platforms combined with adapted clamps can be used alternatively.

Safety remarks

Most organic products are hazardous and need to be handled with care under a working fume hood. The silane solution is corrosive and should be handled with care. Special safety measures should be taken during the preparation of the sulfuric acid/hydrogen peroxide solution (piranha solution), which is described in section “Cleaning of coverslips prior to silanization,” below.

Cleaning of coverslips prior to silanization

Coverslips need to be cleaned and free of organic residues before silanization. The coverslips (~25) were briefly rinsed in acetone and sonicated in a classical ultrasonic bath (sonicator FS6; Fisher Scientific, Illkirch, France) for 20 min in 50% methanol/water, then for an additional 20 min in chloroform, and then air-dried. They were then placed without a special rack in a chloroform-cleaned, dry glass beaker.

We routinely use two different common methods for further extensive cleaning, piranha solution and plasma cleaner, which are described below. A third alternative is cleaning with a UV-ozone cleaner.

Piranha solution. Preparation of the piranha solution is performed under

a fume hood. As its name suggests, this is a very aggressive mixture that is potentially unstable if used carelessly. Therefore, the operator should wear appropriate laboratory protections (lab coat, latex gloves, and safety glasses). Before preparation of the solution, glassware and the working surface were rinsed with chloroform and dried to eliminate organic residues and water, since piranha solution is explosive in contact with organic solvents. Piranha solution (50 mL) was prepared with 70% sulfuric acid (99.999% w/v) and 30% hydrogen peroxide (35% w/v) by first pouring the peroxide solution into a beaker containing the coverslips, which itself was positioned in a bigger beaker containing warm water (~60°C) to control the temperature of the piranha solution. Then, the acid was slowly poured into the beaker. The reaction is exothermic and small bubbles appear indicating the start of the oxidation reaction. Mixing and further heating are not required. After incubation for 20 min in the piranha solution, the coverslips were recovered with Teflon-coated tweezers (one should avoid tweezers made of other polymers or nickel as piranha solution reacts with them) and sonicated for 20 min, first in chloroform, then in ultrapure water. Shorter cycles of sonications in chloroform, followed by those in water, were repeated until the coverslips were totally devoid of any viscous acid residue. After a last sonication in chloroform, the dried coverslips were

silanized overnight. For disposal, the piranha solution was slowly diluted in 3–4 L of water and neutralized with 30% NaOH.

Exposure to plasma. An alternative to the piranha treatment is the cleaning with a plasma cleaner, which is a common apparatus in physics laboratories or an UV-ozone cleaner. The coverslips were briefly rinsed in acetone and sonicated, first 20 min in 50% methanol/water, then 20 min in chloroform. Once dry, coverslips were kept in a dust-free container until treated in a plasma cleaner PCD-002 (Harrick Plasma, Ithaca, NY, USA). Both sides of the coverslips were exposed to the plasma for 1 min. Coverslips were placed in a beaker and then totally dehydrated at >100°C for at least 1 h in an oven.

Overnight silanization

One hundred microliters of (7-octen-1-yl)trimethoxysilane were diluted in 100 mL n-heptane. Once opened, silane vials were stored in a desiccator without vacuum for no more than 2 months (after 1 month, we have sometimes observed a decrease in the quality of DNA fiber alignment). Alternatively, silane can be stored in argon or nitrogen to avoid oxidation and polymerization. Dried coverslips were rapidly transferred into the silane solution without rehydration and kept overnight in a desiccator, without vacuum, under a chemical hood.

Post-silanization cleaning

After silanization, coverslips were transferred into n-heptane and sonicated for 5 min. Coverslips were transferred one by one into water and sonicated for another 5 min in distilled water. Coverslips were recovered and dried before a 5-min final sonication in chloroform. Dry coverslips were kept in a carefully closed container. They can be successfully used for molecular combing of DNA for 2–3 months after silanization. To test the hydrophobicity of the silanized coverslips, a drop of water on a silanized coverslip will form a characteristic round drop, unlike on an untreated surface.

Molecular combing and DNA detection

λ phage DNA was denatured for 15 min at 50°C, immediately put on ice and suspended at 5 $\mu\text{g}/\mu\text{l}$ in 400 μl TE buffer pH 6.5, 0.3 μl YOYO-1 1 mM (Molecular Probes, Invitrogen, Abingdon, Oxon, UK). After dilution to 0.33–1 $\mu\text{g}/\mu\text{l}$ in 1.5 mL 50 mM MES buffer pH 6.1, λ DNA was combed onto a silanized surface. The optimal pH can vary between pH 5.8 and 6.2 depending on the batch of coverslips and on the type of DNA. DNA from *Xenopus* sperm nuclei was replicated in *Xenopus* egg extracts in the presence of 20 μM biotin-dUTP (Roche Applied Science, Meylan, France) (6). DNA of cultured HeLa cells was labeled *in vivo* by 100 μM BrdU for 7 h, then extracted before molecular combing was performed as described (4,10). Fluorescent detection and signal amplification were carried out using AlexaFluor antibodies (Molecular Probes, Invitrogen), mouse anti-BrdU FITC antibody (Becton Dickinson, Le-Pont-de-Claix, France), and Texas Red-conjugated avidin and biotinylated anti-avidin (Vector Laboratories, ABCYS, Paris, France).

Hybridization of probes on combed DNA

Restriction fragments of cloned ribosomal DNA were used as probes on combed genomic DNA, both from HeLa cells and *Xenopus* sperm nuclei (23) (see Results). Fragments

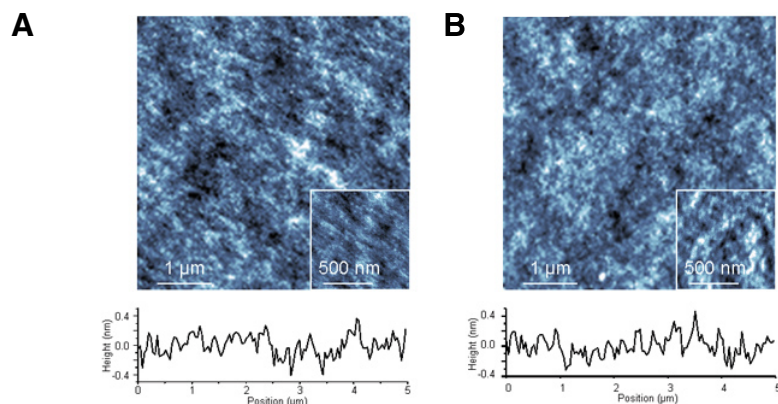


Figure 2. AFM imaging on silanized glass surfaces. Contact mode AFM images of glass surfaces (A) before and (B) after silanization treatment. $5 \times 5 \mu\text{m}$ images show the homogeneous roughness of surfaces in both cases. Curves indicate the height profile in nm of the surface along one line of the image.

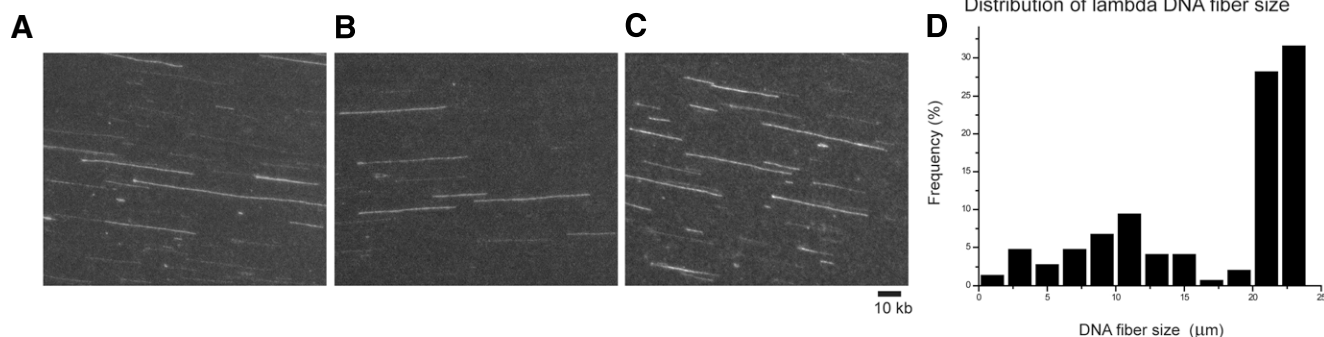


Figure 3. DNA combing of λ phage DNA on silanized coverslips obtained by liquid-phase silanization. λ phage DNA stained with YOYO-1 was combed onto silanized coverslips and immediately visualized by fluorescence microscopy. Stained DNA was diluted in 50 mM MES buffer at different pH: (A) 5.74, (B) 6.04, and (C) 6.2 and combed. (D) Size distribution of 149 λ DNA; bin size, 2 μ m.

were biotinylated using the BioPrime DNA Labeling System (Invitrogen), according to the manufacturer's protocol. Hybridizations were carried out using 200 ng of probes per slide as described (10,24).

Briefly, combed DNA was dehydrated successively for 5 min each in 70%, 90%, and 100% cold ethanol. The rDNA probes were added to the hybridization mix consisting of 50% formamide, 2 \times SSC, 0.5% SDS, 0.5% N-lauryl sarcosyl, 0.3 \times blocking solution [1% blocking reagent, (Roche Applied Science), in 1 \times PBS] (25). After denaturation of both the probes and the combed DNA (see Results), hybridization was performed overnight at 37°C. Slides were washed twice in 50% formamide/2 \times SSC, three times in 2 \times SSC, and once in PBS. Fluorescent detection and signal amplification were carried out using the appropriate fluorochrome-conjugated antibodies (see Results). We found that coverslips pre-cleaned with piranha solution

showed better FISH efficiencies than those that were cleaned by the plasma cleaner.

Atomic force microscopy (AFM) imaging on silanized surfaces

AFM images were performed using a Pico SPM microscope (Molecular Imaging, Phoenix, AZ, USA) in contact mode with silicon nitride cantilevers (spring constant of 0.2 N m⁻¹) (Nanoworld, Neuchâtel, Switzerland) in an N₂ atmosphere.

Imaging and DNA fiber measurements

Images of combed DNA fibers were acquired using a 100 \times , 1.4 numerical aperture UPlanSApo objective on an Olympus IX 81 inverted microscope (Olympus France, Rungis, Val-de-Marne, France) connected to a CoolSNAP HQ CCD camera


(Photometrics, Pleasanton, CA, USA) run by MetaMorph version 6.3r7 (Molecular Devices, Roper Scientific, Evry, France). Images were processed for brightness and contrast with Adobe Photoshop CS2 (Adobe Systems Inc., San Jose, CA, USA). Measurements of combed fibers were made using Image J (National Institutes of Health, Bethesda, MD, USA).

RESULTS

Trimethoxy-octenylsilane is well adapted for molecular combing of DNA when using liquid-phase silanization

DNA fibers can be stretched on any hydrophobic surface with varying efficiency (21). We combed λ phage DNA stained with YOYO-1 on a non-treated glass surface (Figure 1B) and on a polystyrene-coated surface (Figure 1C). On the glass surface, DNA was fixed, as evidenced by bright dots, but was rarely stretched. On the polystyrene-coated surface, DNA fibers were aligned, but with a low density. Importantly, DNA fibers combed on these surfaces were not irreversibly fixed and treatment with denaturing chemicals induced major DNA fiber loss (data not shown).

Silanization is the best known treatment to ensure irreversible fixation and alignment of DNA fibers onto a surface (1). A mono- or multilayer of silane molecules is grafted onto



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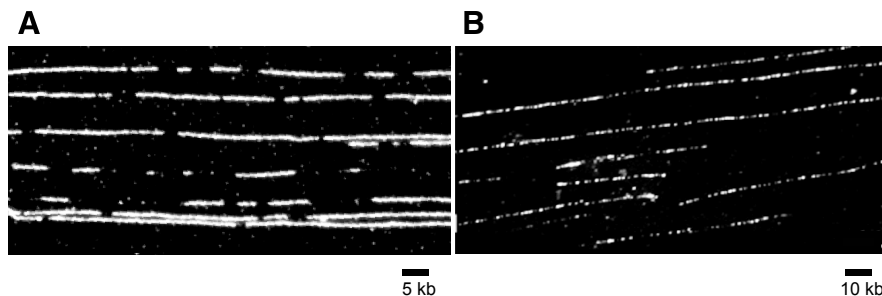


Figure 4. Visualization of combed replicated DNA on silanized coverslips. (A) *Xenopus* sperm nuclei were incubated in *Xenopus* egg extracts. After 40 min, 20 μ M biotin-dUTP was added, and reactions was stopped at 120 min. DNA was extracted; combed; denatured in 50% formamide, 2 \times SSC; and detected with Texas-Red conjugated avidin and biotinylated anti-avidin. (B) Combed BrdU-labeled DNA from HeLa cells was denatured in 2.5 M HCl, detected with a mouse anti-BrdU antibody followed by an anti-mouse AlexaFluor488 antibody.

the glass coverslip exposing a vinyl group. We treated precleaned glass coverslips with two different octenylsilanes and tested DNA combing (see Materials and Methods). λ phage DNA was stained with YOYO-1 and combed with a high density on both trimethoxy-octenylsilane (Figure 1D) and trichloro-octenylsilane (Figure 1E) coated surfaces. The trimethoxy-octenylsilane coated surface showed better DNA fibers alignment and stretching than the trichloro-octenylsilane, which is generally used for gas-phase silanization (1). We did not observe a difference in combing characteristics between coverslips precleaned with piranha solution or treated with the plasma cleaner. However, FISH results (see “Hybridization of probes on combed DNA”) were more reproducible using coverslips precleaned with piranha solution.

We conclude that the trimethoxy-octenylsilane is better adapted in our liquid-phase procedure to achieve optimal DNA combing.

Liquid-phase silanization leads to homogeneously modified surfaces

Common liquid-phase silanization methods produce heterogeneously modified surfaces (26), which result in poor DNA fiber density and attachment. Therefore, we characterized our surfaces by atomic force microscopy before and after silanization with trimethoxy-octenylsilane (Figure 2, A and B). We did not detect domains and therefore could not conclude whether mono- or multilayers of silane molecules were present. The molecular arrangement seems to be disordered.

However, the numerically calculated root-mean-squared roughness for 5 μ m \times 5 μ m images yielded 0.160 nm for the glass surface and 0.162 nm for the silane-treated surface. These two nearly identical values indicate that the root-mean-squared roughness of the glass surface is not modified by the chemical treatment. Moreover, we did not observe silane aggregates to be physisorbed on the treated coverslips. The calculated roughness value on our silanized surfaces was very similar to roughness values obtained for hydrophobic surfaces created by vapor deposition process (26). The small size difference between the solvent n-heptane and the reactant trimethoxy-octenylsilane creates a competition between the two molecules to reach the surface. Therefore the formation of the silane brush on the surface no longer follows a reaction-limited process but a diffusion-limited reaction. The reaction kinetics in this situation approaches the kinetics of a gas phase reaction and might explain the homogeneity of the modified surface.

We conclude that surfaces remain smooth after a silanization process that creates large homogeneous domains at the micrometric scale. We then further characterized the DNA combing on surfaces silanized by the described liquid-phase method for DNA combing.

Liquid-phase silanization produces high quality coverslips with properties similar to surfaces obtained by gas-phase silanization

We combed λ phage DNA stained with YOYO-1 diluted in 50 mM MES

of various pH (Figure 3, A, B, and C) on silanized coverslips. First, we observed that slight variations in pH did not dramatically alter DNA alignment, as stretched molecules could be observed with pH 5.74, 6.04, or 6.2. At pH 6.04, fibers were generally straighter than at pH 5.74 or pH 6.2. In general, better results were obtained with a less acidic (\sim pH 6.0) buffer than one used for combing on gas-phase silanized surfaces using trichloro-octenylsilane (\sim pH 5.5) (1).

Regardless of pH, we consistently observed some surface regions with less straight DNA fibers. Thus, some heterogeneities on the surfaces cannot be totally avoided when silanizing in liquid-phase, but those heterogeneous spots are sparse and easily distinguishable. Minor differences between independent batches of silanized coverslips have been observed, but the described method generally gives good, reproducible results. If more than 20% of the surface showed inhomogeneous or non-parallel fiber stretching, the coverslip or the entire batch was not used further.

We next measured standard DNA in order to determine the stretching factor on our surfaces. Figure 3D shows the size distribution of 149 combed λ phage molecules. Even though the DNA solution was carefully prepared to avoid DNA shearing, some broken molecules were observed. A significant peak was observed between 21 and 24 μ m. The width of this peak can be explained by the breaking of some molecules and the imprecision of the measurements. Longer molecules than the theoretical length could be explained by rare concatemers. We next averaged the

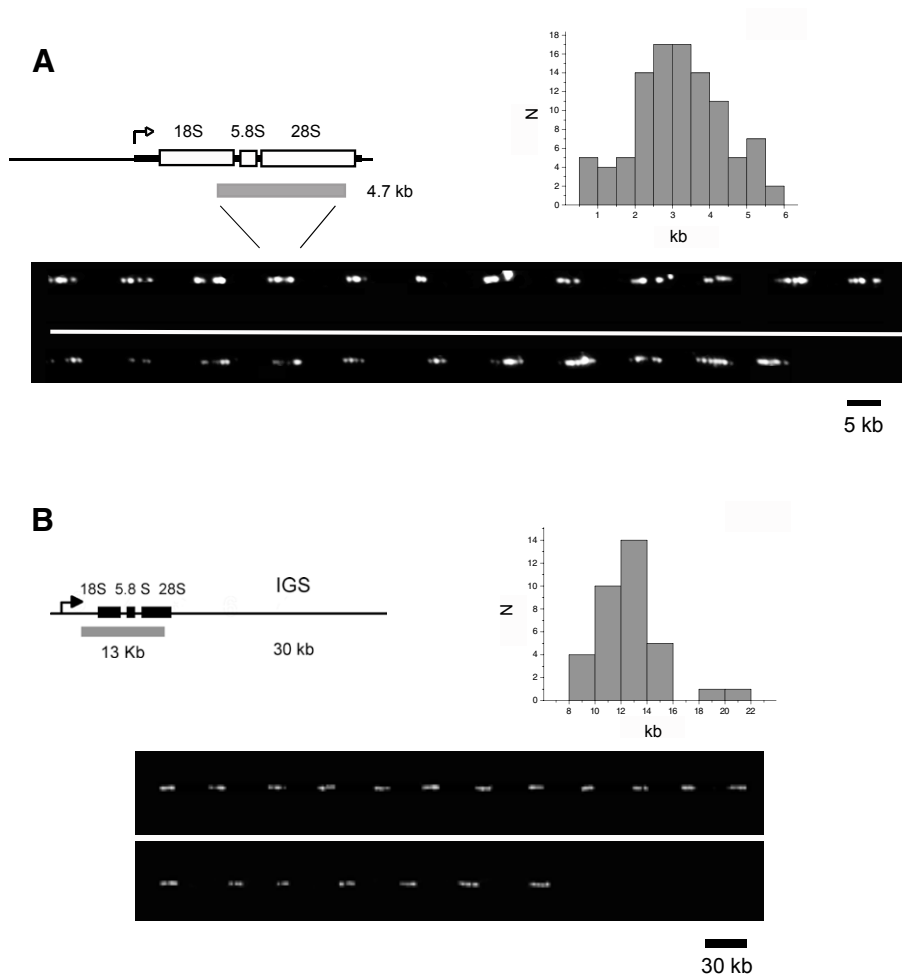


Figure 5. FISH on combed *Xenopus* and human rDNA sequences. rDNA biotinylated probes were hybridized on combed DNA fibers from (A) *Xenopus* sperm and (B) HeLa cell DNA. Each panel shows the map of the rDNA repeat unit and the probe (gray rectangle), two images of hybridized rDNA tandem repeat units, and a histogram of measured probe sizes in kb.

size of the 75 molecules longer than 21 μm and compared their values to the length of λ phage DNA (48,502 bp), thereby allowing us to determine an equivalence between physical distance and genomic length of 1 μm \sim 2.2 kb, with a 5% length variation depending on experiment, coverlip variations, etc. We concluded that the equivalence between physical distance and genomic length of DNA on liquid-phase silanized coverslips is very similar to the one determined with gas-phase silanized coverslips (1 μm \sim 2.0 kb) (1).

Surfaces are suitable for the detection of replication eyes, forks, and fluorescent in situ hybridization (FISH) on combed fibers

Next, we tested whether the combed DNA on the silanized coverslips could

remain resistant, without major fiber loss, to the various treatments involved in the visualization of combed DNA other than staining with YOYO-1. Such treatments require denaturation either using formamide, NaOH, HCl, or heating of the stretched double-stranded DNA, in order to facilitate the access of antibodies or DNA probes.

We first combed DNA from *Xenopus* sperm nuclei labeled by in vitro replication with biotin-dUTP (6). Nuclei were incubated in *Xenopus* egg extracts. After 40 min, at which time S phase had started, 20 μM of biotin-dUTP were added, and the reaction was stopped at 120 min, as described in Reference 8. Combed DNA was denatured in 2 \times SSC, 50% formamide at 70 $^{\circ}\text{C}$ for 2 min. Biotin-labeled DNA was revealed with the appropriate antibodies: Texas Red-conjugated avidin and biotinylated

anti-avidin (Figure 4A). In this labeling protocol, replication eyes are defined as regions replicated before the addition of biotin-dUTP and appear as gaps in a biotin-labeled DNA fiber. Next, DNA from HeLa cells, labeled in vivo for 7 h with BrdU, was combed, denatured with 2.5 M HCl for 60 min, and then immunostained by mouse anti-BrdU antibody-FITC followed by a goat anti-mouse AlexaFluor488 antibody (Figure 4B). Immunostainings of labeled DNA showed good quality; the fiber density did not indicate a major fiber loss after different denaturation treatments. Denaturation with 1 N NaOH gave similar results (data not shown).

Finally, we carried out FISH on *Xenopus* and human DNA, combed on silanized coverslips (Figure 5). FISH requires extensive denaturation in order to allow base pairing between the stretched DNA and the labeled probes. DNA from *Xenopus* sperm nuclei was combed and co-denatured with a biotinylated rDNA probe for 5–8 min at 75–80 $^{\circ}\text{C}$ in the presence of 50% formamide in the hybridization mix. Hybridization was carried out, as described in Reference 10, and the biotinylated probe on combed DNA was detected with AlexaFluor 594 streptavidin and biotinylated anti-streptavidin. *Xenopus* rDNA repeats could be visualized (Figure 5A) with a satisfactory frequency per coverslip (depending on DNA fiber density: 30–100 fibers containing rDNA repeats per coverslip), indicating a good hybridization efficiency; measurements on multiple repeats confirmed the known length (Figure 5A, histogram) (23,24). A similar FISH experiment was undertaken using DNA from HeLa cells to detect human rDNA sequences. Here, combed DNA was denatured for 30 min in 1 M NaOH and FISH was performed, as described in Reference 25. Human rDNA repeats (Figure 5B) were visualized with good frequency per coverslip. Measurements of the probe sizes provided the expected values (Figure 5B, histogram) (27).

We conclude that the silanized coverslips produced by this optimized liquid-phase silanization method are very suitable for most of the applications of molecular combing, such as detection of

different incorporated modified nucleotides and FISH.

DISCUSSION

Molecular combing of DNA requires homogeneously silanized surfaces that will ensure the formation of a high-density array of straight DNA fibers. Previously described protocols use gas-phase silanization, which is technically demanding (1). Thus, we developed and optimized a much simpler protocol based on liquid-phase silanization using a novel combination of silane/solvent which is 0.1% (7-octen-1-yl)trimethoxysilane in n-heptane.

Silanized coverslips obtained with this liquid-phase preparation possess all the properties required for successful combing of the DNA. DNA molecules are stretched reproducibly so that 1 μm of molecule length is ~ 2 kb. Fixation of DNA fibers is irreversible and resistant to all treatments, especially denaturation, used for the detection of incorporated modified nucleotides and FISH.

When conducted under the appropriate conditions, liquid-phase silanization has proven to be a suitable and reproducible alternative for gas-phase silanization. Besides extensive pre-cleaning steps prior to silanization, the choice of the right silane/solvent combination is probably one of the critical factors.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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