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Microfluidic extraction and stretching of chromosomal DNA from single cell nuclei for DNA fluorescence *in situ* hybridization

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Abstract

We have developed a novel method for genetic characterization of single cells by integrating microfluidic stretching of chromosomal DNA and fiber fluorescence *in situ* hybridization (FISH). In this method, individually isolated cell nuclei were immobilized in a microchannel. Chromosomal DNA was released from the nuclei and stretched by a pressure-driven flow. We analyzed and optimized flow conditions to generate a millimeter-long band of stretched DNA from each nucleus. Telomere fiber FISH was successfully performed on the stretched chromosomal DNA. Individual telomere fiber FISH signals from single cells could be resolved and their lengths measured, demonstrating the ability of the method to quantify genetic features at the level of single cells.

Keywords

Fluorescence *in situ* hybridization; DNA; Single cell; Microfluidic; Fiber FISH; Telomere

1 Introduction

Fluorescence *in situ* hybridization (FISH) is widely used to detect and quantify specific nucleic acid sequences in cell genomes for both fundamental research and clinical diagnosis (Wolff and Schwartz 2004). FISH is typically performed on interphase and metaphase cells, in which chromosomal DNA is highly condensed by nuclear proteins. As a result, the location, quantity, and size of a target sequence are characterized at a resolution of approximately 2–4 megabase pairs (Mbp) (Wolff and Schwartz 2004). The resolution can be significantly improved to one kilobase pair (kbp) if FISH is performed on chromosomal DNA molecules stretched on a solid surface (Lebofsky and Bensimon 2003). This technique, called fiber FISH, is useful for mapping genes, studying DNA replication, and assessing cancer-related genetic mutations.

Several methods have been developed for extracting and stretching chromosomal DNA. In one, approximately one million cells are embedded in an agarose gel block (Michalet et al. 1997). Protease is allowed to diffuse into the gel to degrade the proteins and consequently free the chromosomal DNA. Degrading the gel with agarase releases the DNA molecules into solution, and they are then stretched onto a solid surface through a dewetting process. In a second method, free chromosomal DNA molecules are also generated in a gel block placed on a glass slide (Heiskanen et al. 1996), but rather than being digested with an enzyme, the gel is melted to release the DNA. Simultaneously, a shear flow is generated to stretch the DNA molecules. In a variation of this method, chromosomal DNA is first released from approximately 2000 cells in a solution on a glass slide (Jackson and Pombo 1998). The slide is then tilted to allow the solution to flow, stretching the DNA on the slide. In a third method, a solution of DNA molecules is allowed to flow through a microscopic channel with a positively charged floor, on which the DNA molecules are immobilized and stretched (Dimalanta et al. 2004).

Fiber FISH has been successfully performed on the stretched DNA generated by the first two methods, but in all three methods, the DNA fibers and corresponding FISH signals could not be traced back to the cells from which they originated. Doing so can be essential for certain applications such as quantifying intercellular genetic heterogeneity, which is thought to play an important role in cancer drug resistance and relapse (Merlo et al. 2006). Moreover, these methods require large numbers of cells, whereas for certain applications such as characterization of circulating cancer cells and prenatal diagnosis of inherited diseases, only a small number of cells or even a single cell is available. Attempts have been made to pull DNA from single cells by electric field, and FISH is performed on the stretched DNA (Shaposhnikov et al. 2009), but this technique, called comet assay, is useful only when a large number of double-strand breaks is present in the chromosomal DNA. Similarly, electro-osmotic flow is employed to pull DNA from a single cell (Hung et al. 2009) and a single metaphase chromosome (Hung and Chen 2010), but the DNA is not well stretched and fiber FISH is not demonstrated with either of these methods.

Here, we describe a new technique that overcomes the limitations of the above methods. It used pressure-driven microfluidic flow to extract and stretch chromosomal DNA from single, identifiable cells. The DNA was well stretched, and high-resolution fiber FISH of telomeres was successfully performed.

2 Experimental

2.1 Materials and reagents

Glass slides, cover slips, tubing, formamide, formaldehyde, TE buffer at pH of 8.0, phosphate buffered saline (PBS), saline-sodium citrate (SSC) buffer, and herring sperm

DNA were purchased from VWR. Colchicines, sodium salt of poly(acrylic acid) (PAA), 3-aminopropyltriethoxysilane (APTES), poly(allylamine hydrochloride) (PAH), bovine serum albumin (BSA), and TE buffer were purchased from Sigma-Aldrich. Polymerase chain reaction (PCR) primers (TTAGGG)₃ and (CTAACC)₃ were purchased from IDT (Coralville, IA, USA). Sylgard 184 poly(dimethyl siloxane) (PDMS) was purchased from Dow-Corning. Poly(methyl methacrylate) (PMMA) plate was purchased from McMaster-Carr (Robbinsville, NJ, USA). Plastic connectors were purchased from Value Plastics (Fort Collins, CO, USA). YOYO-1 fluorescence dye, human cot-1 DNA, BioNick™ DNA Labeling System, rhodamine-labeled neutravidin, pepsin Digest-All™, and ProLong® Gold antifade reagent containing 4',6-diamidino-2-phenylindole (DAPI) were purchased from Invitrogen. DIG-Nick Translation Mix and anti-digoxigenin-fluorescein fab fragments were purchased from Roche. Proteinase K was purchased from New England Biolabs. Mouse embryonic stem cell line ESD3 was from Joy Rathjen at the Department of Zoology, University of Melbourne, Australia.

2.2 Methods

The process of this new technique is shown schematically in Fig. 1. First, single interphase cell nuclei were immobilized in a microchannel. Each nucleus was then exposed to protease solution, which released the chromosomal DNA. A pressure-driven flow was generated to stretch the released DNA. Finally, FISH was performed on the stretched DNA. The experimental setup of the system is shown in Fig. 2(a). It consisted of a microfluidic chip, tubing, a syringe, a syringe pump, and a liquid collector. The microfluidic chip encompassed a glass slide and a PDMS slab. The slide was coated with APTES for immobilization of cell nuclei and DNA. The PDMS slab was pierced by two holes, and a straight open channel connected the two. Bonding the slide (on which nuclei had been deposited as described below) and the PDMS slab together sealed the channel. Each end of the channel was connected, through the hole, to a piece of tubing. One tube was coupled to the syringe and pump, and the other led to the liquid collector. Liquids were first loaded into the tubing between the syringe and the chip and later driven into the microchannel by the pump.

2.2.1 Fabrication of PDMS slab

A computer numerical control (CNC) mill system (Sherline Products, Vista, CA, USA) was used to carve a straight channel 250 μm deep, 1 mm wide, and 2 cm long and a circular hole 1.5 mm in diameter at each end of the channel, in a 1.5-mm-thick PMMA plate. The plate was then used as a master for casting a PDMS mold (Xia and Whitesides 1998). The mold, featuring a straight ridge with a post at each end, was sputter coated with a 10-nm-thick layer of gold. The two posts were inserted into two 5-cm pieces of plastic tubing. The gold-coated, tubing-connected mold was used to cast the final PDMS slab, which featured a straight channel of virtually the same dimension as the original channel in the PMMA master, and the two pieces of tubing were firmly embedded in the final PDMS slab, connected to the channel. The slab was then cut to 2 cm in width and 4 cm in length.

2.2.2 Coating of glass slides with APTES

Glass slides 6 cm long, 2.5 cm wide, and 0.17 mm thick were first exposed to oxygen plasma in a PDC-32 G plasma cleaner (Harrick Plasma, Ithaca, NY, USA) and then immediately placed in a vacuum desiccator with 100 μL APTES in a 1.5-mL centrifuge tube. After vacuum was created in the desiccator by a pump (Welch Model 8907), the desiccator was moved to an oven at 65°C. The glass slides were taken out after 48 h and rinsed with deionized water before use.

2.2.3 Deposition of cell nuclei on APTES slide

Nuclei were extracted from the mouse embryonic stem cells by treatment with hypotonic KCl solution (Sivak and Wolman 1974) then suspended in 3:1 methanol:acetic acid fixative. To deposit the nuclei on an APTES-coated glass slide, we placed an unmodified 1-mm-thick glass microslide on the APTES slide to form a step running across the width of the APTES slide. A small volume (~100 μ L) of nucleus suspension was dropped at one end of the step and was spontaneously drawn by capillary action to the other end along the step. The solvent in the suspension evaporated rapidly, leaving a row of nuclei roughly aligned across the APTES slide.

2.2.4 Stationary release of chromosomal DNA

This experiment provided well-controlled flow-free conditions for releasing chromosomal DNA and was not part of the microfluidic stretching of the DNA. A 1-cm-wide square hole was cut through a piece of PDMS slab 3 mm thick. The PDMS slab was placed on an APTES-coated glass slide to form a well. The methanol and acetic acid in the nucleus suspension were replaced with PBS by centrifugation and resuspension. The nucleus suspension in PBS was then placed in the PDMS well for 10 min before being aspirated, leaving approximately 20 cell nuclei on the slide. Then, 200 μ l 100 μ g/mL proteinase K and 0.01 wt% PAA in TE buffer were added to the well. A trace amount of YOYO-1 was also added to stain chromosomal DNA. The nuclei were imaged after 1 h of incubation at 25°C.

2.2.5 Assembly of microfluidic chip

We assembled the microfluidic chip for extracting and stretching chromosomal DNA from nuclei by placing the channel-bearing PDMS slab on an APTES-coated slide carrying deposited nuclei. The PDMS slab adhered to the surface spontaneously, typically tightly enough to prevent leaks during the DNA-stretching phase. Usually 10 to 20 nuclei were enclosed in the microchannel. A length of tubing was loaded with measured volumes of appropriate liquids as illustrated in Fig. 2. One end of this tubing was then connected to the short inlet tubing embedded in the PDMS slab. The other end was connected to a water-filled 10 or 50 mL syringe mounted on a syringe pump (KD Scientific). Plastic connectors were used to connect the tubing and the syringe.

2.2.6 Microfluidic extraction and stretching of chromosomal DNA

To extract and stretch chromosomal DNA from the nuclei in the microchannel, we injected the liquid in the tubing into the microchannel at controlled rates driven by the pump. The flow rate either remained constant throughout the experiment or changed once at a predetermined point during the experiment. First, the proteinase K solution released the chromosomal DNA by degrading nuclear proteins. Once the DNA had been released and stretched, a polycation, PAH, was employed to fix it. Three different combinations of liquids and flow conditions were used, as shown schematically in Fig. 2(b–d), the rationales for which are described below under Results and Discussion. In Method 1 (Fig. 2 (b)), a relatively high concentration of proteinase K (100 μ g/ mL) and a constant, relatively low flow rate (1 mL/h) were used. In contrast, in Method 2, a relatively low concentration of proteinase K (0.4 μ g/mL) and a relatively high flow rate (5 mL/h) were used (Fig. 2(c)). In addition, a polyanion, PAA, was added to the proteinase K solution in Method 2. In Method 3, three different types of solutions and two different flow rates were used (Fig. 2(d)). The 1 mL volume of the proteinase K solution and 1 mL/h flow rate allowed 1 h for DNA release and was followed by a relatively rapid flow of water (20 mL/h). YOYO-1 dye was added to the proteinase K solutions in all three methods, to label the DNA.

2.2.7 FISH

Biotinylated telomere FISH probes were prepared by PCR and nick translation of the PCR product. The DIG-Nick Translation Mix was used to produce probes for interphase and metaphase FISH. The BioNick™ DNA Labeling System was used to produce the probes for fiber FISH. Oligonucleotides (TTAGGG)₃ and (CTAACC)₃ were used as primers for PCR, and the amplification procedure developed by Ijdo et al. (1991) was used. A protocol developed by Lansdorp et al. was followed to perform metaphase and interphase telomere FISH (Lansdorp et al. 1996). The anti-digoxigenin-fluorescein fab fragments were used for fluorescence staining. For fiber FISH, we (1) removed the PDMS slab from the glass slide; (2) baked the slide at 65° C in air for 2 h; (3) soaked the slide in PBS for 15 min; (4) soaked the slide in 4% formaldehyde for 10 min; (5) rinsed the slide three times with PBS for 5 min each; (6) placed the slide to denaturing solution (70% formamide in 2× SSC buffer) at 95°C for 15 min; (7) dehydrated the slide in a 70%, 90%, 100% ethanol series at −20°C for 5 min each and air-dried it; (8) added the denatured probe (75°C for 10 min) to the slide, covered it with a plastic coverslip, and incubated the slide at 37°C for 12–16 h; (9) rinsed the slide in 2× SSC solution three times at 45°C for 5 min each; (10) added 100 μL blocking solution (3% BSA/4× SSC/2% Tween 20) to the slide and covered it with a glass coverslip; (11) incubated the slide at 37°C for 30 min; (12) removed the cover slip and added 25 μL rhodamine-labeled neutravidin solution (20 mg/mL in 1% BSA/4× SSC/2% Tween-20) to the slide and covered it with a glass coverslip; (13) incubated the slide at 37°C for 30 min; (14) rinsed the slide three times with 2× SSC for 5 min each and air-dried it in the dark; (15) mounted the slide in 10 μL antifade reagent and covered it with a glass coverslip.

2.2.8 Imaging

Fluorescence imaging was performed on a Nikon Ti-U inverted fluorescent microscope equipped with an Andor iXon^{EM}+885 EMCCD camera and a×100 oil immersion objective with 1.3 numerical aperture.

3 Results and discussion

3.1 Analysis of flow field and shear stress

Stretching the DNA in a repeatable manner required creating a stable laminar flow in the microchannel. The Reynolds number for the channel, Re , was calculated as

$$Re = v_{av} h \rho / \mu,$$

where v_{av} was the average linear axial velocity, h the height of the channel, ρ the density of the buffers, and μ the viscosity of the buffers. v_{av} was calculated as

$$v_{av} = Q / hw,$$

where Q was the volumetric flow rate; h the height of the channel; and w the width of the channel. For the microchannel, which was 1 mm wide and 250 μm high, v_{av} was 22.2 mm/s at the highest volumetric flow rate of 20 mL/h. On the assumption that the densities of the buffers were 1 g/mL and their viscosities 1×10^{-3} Pa·s, Re was 5.6 at a flow rate of 20 mL/h. This value is much smaller than the threshold, 9200, for the channel aspect ratio of 4 (2886 for plane Poiseuille flow) (Tatsumi and Yoshimura 1990), required for transition to turbulence. The flows we used therefore fall well within the laminar regime.

Because the cell nuclei were fixed to the floor of the microchannel, we believe that wall shear stress in the vertical direction on the channel floor was responsible for stretching the DNA, but the wall shear stress was not uniform across the width of the channel because of the presence of the two side walls. Because multiple cell nuclei were placed across the width of the channel, the effects of the side walls on the wall shear stress had to be elucidated. The shear stress distribution for Poiseuille flow in a rectangular channel is detailed by Boussinesq (1868) and White (1974) and can be expressed in terms of the deviation from that in an infinitely wide channel (plane Poiseuille flow). The wall shear stress in the plane Poiseuille flow τ_{wall}^{∞} was calculated as 6.33 dynes/cm² (see Appendix for details). The wall shear stress τ_{wall} in the channel was normalized by τ_{wall}^{∞} and is plotted against channel width in Fig. 3 (see Appendix for details), which shows that the shear stresses are above 90% of τ_{wall}^{∞} within the region ~0.17 mm from the side walls. All cell nuclei for DNA stretching fell into this region, so the side walls exerted insignificant effects on the stretching of the DNA, but the variation in the wall shear stresses along the channel width within this region is expected to contribute to the differences in lengths of the stretched DNA bands from different cell nuclei.

3.2 Three methods for stretching chromosomal DNA

Our objective was to develop a microfluidic technique capable of extracting and stretching chromosomal DNA from single cell nuclei for fiber FISH. Because several factors were found to affect the pattern of stretched DNA, we investigated three different combinations of liquid type and flow conditions to identify the optimal conditions for fiber FISH.

Conditions common to all three included the APTES-coated microchannel floor and the use of proteinase K and PAH in the solutions. APTES coating is widely employed for immobilizing DNA by offering a positive surface charge (Liu et al. 2005). We used it to immobilize not only released DNA but also the nuclei, because mammalian cell nuclei are negatively charged at neutral pH (Badr and Waldman 1973). Figure 4(a) shows a single cell nucleus, approximately 8 μ m wide, immobilized on an APTES-coated slide before its DNA was released. Proteinase K is widely used to release chromosomal DNA by degrading nuclear proteins (Michalet et al. 1997). We demonstrated its effect by statically releasing chromosomal DNA from a single nucleus to form a significantly enlarged, roughly globular structure as shown in Fig. 4(b). PAH is a polycation commonly employed in layer-by-layer assembly (Clark and Hammond 2000). We used it to fix the negatively charged, stretched DNA to the channel floor. Moreover, because PAH carried a high density of primary amines, formaldehyde treatment in the FISH process might have cross-linked PAH molecules and APTES to further fix the stretched DNA to the substrate (Lansdorp et al. 1996).

The rationale for the ways in which Method 1 differed from the others—constant, relatively low flow rate; relatively high concentration of proteinase K; and absence of PAA in the DNA releasing and stretching solution—was to release the DNA gradually and simultaneously to stretch the released molecules. This method generated a spindle-shaped pattern of DNA released from a single nucleus as shown in Fig. 4(c). The widest portion of the pattern was approximately 61 μ m wide, and the total length was about 218 μ m. We hypothesize that the relatively high concentration of proteinase K led to rapid degradation of the nuclear proteins and therefore fast release of the chromosomal DNA. Because the released DNA expanded radially in the absence of flow, as shown in Fig. 4(b), this tendency competed with the directional stretching caused by the microfluidic flow, producing the spindle-shaped pattern. The immediate adherence of the released DNA to the APTES-coated surface probably contributed. The relatively short, wide pattern indicated that the DNA molecules were not well stretched. This method was therefore not used for fiber FISH.

To solve this problem, in Method 2, we added PAA to the proteinase K solution; reduced concentration of proteinase K to decrease DNA release rate; and used a constant, relatively high flow rate. We assumed that PAA would bind to the APTES-coated slide, producing a negatively charged surface at pH 8. As a result, adherence of the released DNA to the surface would be inhibited, and the released DNA would not form so short and wide a spindle as in Method 1. The experimental result is shown in Fig. 4(d). The stretched DNA formed a slender, fiber-like structure with uniform fluorescence intensity along its length from each nucleus. The “fibers” ranged from 750 to 1000 μm in length and 0.3 to 0.5 μm in width. Although the greater length indicated that the chromosomal DNA was stretched to a greater extent, the narrow “fibers” would not allow clear discrimination of adjacent FISH signals, so Method 2 was also not considered suitable for fiber FISH.

Method 3 was intended to overcome the drawbacks of the first two methods. Its rationale was to release the DNA first and then to stretch it with a high shear-rate flow. Chain extension of a tethered DNA molecule has been demonstrated to increase with shear rate (Ladoux and Doyle 2000). We used a relatively high concentration of proteinase K (100 $\mu\text{g}/\text{mL}$) in this method to ensure a high degree of DNA release. Although releasing the DNA in the absence of flow would have been preferable, maintaining such conditions in the microchannel for 1 h proved difficult. Spontaneous flow frequently occurred in the microchannel, stretching the released DNA in unpredictable directions. To minimize this problem, we maintained a slow flow (1 mL/h) during the release phase. Under those conditions, the released DNA temporarily formed a spindle-shaped structure similar to that in Fig. 4(c), before the flow rate was increased to 20 mL/h started. The PAA in the proteinase K solution prevented the released DNA from adhering to the APTES-coated surface, allowing the later high flow to stretch the DNA significantly.

The results of Method 3 are shown in Fig. 5. Figure 5(a) displays DNA extracted and stretched from a column of single nuclei. The DNA released from each nucleus formed a band with relatively uniform width along most of its length. Bands ranged from 14 to 33 μm in width, moderately wider than those of the nuclei deposited on the APTES-coated slide but much narrower than the widest portion of the spindle-shaped pattern obtained by Method 1. Straight lines were resolved within the bands, as shown in Fig. 5(b). They may have been single stretched DNA chains or bundles of such molecules, indicating that the DNA was well stretched. Moreover, the bands ranged from 1 to 4 mm in length, much longer than those produced by Methods 1 and 2. Figure 5(c) shows three entire bands of DNA approximately 1.9 mm long. This greater length also indicated that the DNA was better stretched than that produced by Methods 1 and 2.

3.3 Fluidic analysis of DNA stretching

The success of Method 3 raised the question of whether an end-tethered mouse chromosomal DNA can be stretched under the same flow conditions (Ladoux and Doyle 2000). Fractional stretch x is defined here as the ratio of the stretched length to the contour length of a DNA molecule.

It depends on the Weissenberg number, Wi , and is expressed as

$$x = 1 - 1.2Wi^{-1/3},$$

where constant 1.2 is obtained from the slope of published experimental data (Ladoux and Doyle 2000). Wi is expressed as

$$Wi = \dot{\gamma}t,$$

where $\dot{\gamma}$ is shear rate and t relaxation time of the DNA chain. We believe stretching of the chromosomal DNA from single cell nuclei as demonstrated in our study is related to this mechanism, so we calculated Wi . The longest relaxation time t can be calculated as (Klotz and Zimm 1972)

$$t \approx 1.247 \times 10^{-14} M^{5/3} = 6.24 \times 10^{-10} N_{bp}^{5/3} (\text{sec}),$$

where M is the molecular weight of DNA and N_{bp} is the number of base pairs in the DNA chain. The latter equation is obtained using the molecular weight of each base pair of double stranded DNA as 660 Daltons. For the smallest mouse chromosomal DNA molecule of 58 Mbp long (Waterston et al. 2002), this relation yields a relaxation time of about 5423 s. The shear rate $\dot{\gamma}$ is calculated as

$$\dot{\gamma} = \tau_{wall} / \mu,$$

where τ_{wall} is the wall shear stress and μ the viscosity of the buffers. Because all cell nuclei in the channel reside in the region (0.17 mm away from the side walls) where the wall shear stresses are above 90% τ_{wall}^{∞} , those stresses within this region are above 569.7 s^{-1} at 20 mL/h. The minimum Wi within this region is therefore 3.09×10^6 , and the fractional stretch is above 0.99, indicating that the DNA chains can be considered to be completely stretched. Note, however, that each chromosomal DNA molecule was probably not tethered only at one end. Instead, multiple points on a DNA molecule might bind to the channel floor during the stretching. Moreover, the interactions between different chromosomal DNA chains probably complicated the stretching process.

3.4 Telomere fiber FISH

To demonstrate the usefulness of this approach for FISH, we used Method 3 to generate samples for analysis by telomere FISH. A telomere is a DNA-protein complex at the end of a eukaryotic chromosome. It is responsible for chromosomal stability and plays important roles in carcinogenesis and aging (Nicholas et al. 2009). Human telomere DNA consists of a repeat sequence and its length is critical to its functions. Several techniques have been developed for measuring telomere length, including fiber FISH, which allows visualization of telomeres at the level of single DNA molecules with high resolution (Jiang and Gill 2006). In our study, mouse embryonic stem cells were used as a model system. Different types of mouse cells differ in telomere sequence length. One study reported an average length of 39.4 kbp (Zalzman et al. 2010), another that telomeres of wild-type of mouse embryonic stem cells were 50.3 ± 20.0 kbp long (3.25% over 90 kbp; Gonzalo et al. 2006), and a third an average length of 80 kbp in mouse primary cells (McIlrath et al. 2001).

We performed metaphase and interphase telomere FISH to confirm the presence of the telomeres in the cell line. Figure 6(a) shows metaphase FISH signals at the ends of the chromosomes, and Fig. 6(b) shows scattered telomere FISH signals in an interphase nucleus. The result of the fiber FISH is shown in Fig. 6(c). Telomere FISH signals were bright line segments within a dark band. The dark band could be stained by YOYO-1 dye, which stains single-stranded DNA as well as double stranded DNA (Cosa et al. 2001). A single-layer fluorescence staining method (rhodamine-labeled neutravidin on the biotinylated probe), rather than the double-layer method used in many fiber-FISH processes was employed. The

effectiveness of single-layer staining for telomere fiber FISH has been demonstrated by Takemura et al. (2008). Moreover, in a significant portion of cases in our study, the FISH signals were straight and relatively uniform in fluorescence intensity not only along each fiber but also among different fibers, indicating that these signals represented single stretched telomeric tracts. 39 such fiber FISH signals were identified from multiple nuclei, and their lengths were measured. Figure 7 shows a histogram of the number of telomere fiber FISH signals plotted against length of the signals, which ranged from 12.3 to 92.0 μm , with a mean of 34.4 μm . The 34.4- μm length corresponds to a genomic length of 101 kbp, on the assumption of 0.34 nm per base pair for double-stranded DNA. This average telomere length is higher than those reported in the literature, possibly because the DNA molecules were over stretched. Note that overstretching is typical in a commonly used method for stretching DNA (Lebofsky and Bensimon 2003), in which a 1- μm physical length of stretched DNA corresponded to a genomic length of 2 kbp. If this scale is adopted, the 34.4 μm -average length we obtained corresponded to a genomic length of 68.8 kbp. This size is comparable to the reported values, indicating that this method may be useful for precisely quantifying telomere sequences of single cells. More important, successful performance of telomere fiber FISH demonstrates the potential of this new technique for characterizing many other genetic features, such as gene amplification and translocation.

3.5 Strengths and weaknesses of the approach

Technically speaking, one of the most notable strengths of this new technique is the use of pressure-driven microfluidic flow. It permitted not only precise control of flow rate over an extended period of time but also change of flow rate during the course of the procedure. Moreover, it allowed continuous delivery into a microchannel of a series of liquids with different functions, including DNA releasing, stretching, and fixation. This set of capabilities makes this technique superior to the microfluidic approach for DNA stretching based on capillary effect (Dimalanta et al. 2004), but it still suffers from some drawbacks at its current stage of development. Notably, the stretched DNA molecules generated could not be individually resolved as clearly as those produced by other techniques (Lebofsky and Bensimon 2003; Dimalanta et al. 2004). Moreover, the mechanism for DNA immobilization cannot guarantee that all chromosomal DNA molecules will be immobilized on the slide surface, or some might be washed away during DNA release and stretching. In addition, curved and overlapped telomere FISH signals were observed (data not shown), indicating that the DNA chains were not well stretched in some samples. Note that a diploid mouse genome contains 5 billion base pairs, corresponding to a total contour length of 1.7 m (Waterston et al. 2002). The smallest chromosome (chromosome 19) is 58 Mbp in genomic size and 19.7 mm in contour length. These values also reflect that the chromosomal DNA molecules were not fully stretched in our study, but improvements to the procedure might solve these problems, perhaps by ensuring complete release of chromosomal DNA and tethering one end of a chromosomal DNA molecule to the substrate in a microchannel before stretching.

4 Conclusions

A novel microfluidic technique has been created with which to extract and stretch chromosomal DNA from single cell nuclei. Under the most successful conditions, a millimeter-long band of stretched DNA molecules was generated from a single nucleus. Telomere FISH was successfully performed on the stretched DNA. This technique is applicable to other cell types for studying various genetic features. It promises to be particularly useful for genetic characterization when only a handful of cells or even a single cell is available.

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Appendix

The shear stress distribution for Poiseuille flow in a rectangular channel can be expressed in terms of the deviation from that in an infinitely-wide channel (plane Poiseuille flow, Boussinesq 1868, White 1974). The wall shear stress in plane Poiseuille flow is expressed as

$$\tau_{wall}^{\infty} = \frac{6\mu v_{av}^{\infty}}{h} = \frac{6\mu v_{av}}{h(1-\delta)},$$

where

$$\delta = \frac{192}{\pi^5 A} \sum_{n=1,3,5,\dots}^{\infty} \frac{\cosh(n\pi A) - 1}{n^5 \sinh(n\pi A)}.$$

At $\mu = 1 \times 10^{-3} \text{ Pa} \cdot \text{s}$, $h = 250 \mu\text{m}$, and channel aspect ratio $A = w/h = 4$, δ and τ_{wall}^{∞} are calculated to be 0.158 and 6.33 dynes/cm² respectively.

For a channel of width w and height h , the shear stresses normalized by τ_{wall}^{∞} in the channel are given by

$$\tau = (1 - 2z) - \frac{8}{\pi^2} \sum_{n=1,3,5,\dots}^{\infty} \frac{\sinh(n\pi y) + \sinh(n\pi(A - y))}{n^2 \sinh(n\pi A)} \cos(n\pi z),$$

$$0 < y < A = \frac{w}{h} \quad 0 < z < 1$$

where the coordinates along the width and height, y and z , respectively, have been normalized by the channel height h and channel aspect ratio A .

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Fig. 1. Schematic representation of the process for extracting and stretching chromosomal DNA from a single cell nucleus and performing telomere FISH

Fig. 2.

(a) Photograph of the microfluidic system, which consisted of a microfluidic chip, tubing, a syringe, a syringe pump, and a liquid collector. Liquids were loaded into the tubing between the syringe and the chip before being injected into the chip by pressure from the syringe. (b, c, d) Schematic diagrams of Methods 1, 2, and 3 respectively. The square, circle, and horizontal bar in each panel represent the syringe, microfluidic chip, and tubing in between, respectively. The colored segments represent different types of liquids in the tubing. Volumes and flow rates of the liquids are specified. PAA, sodium salt of poly(acrylic acid); PAH, poly(allylamine hydrochloride)

Fig. 3.

Distribution of wall shear stress on the floor of the microchannel plotted against the width of the channel. The stress is normalized by the wall shear stress in plane Poiseuille flow (6.33 dynes/cm²)

Fig. 4. Fluorescence micrographs of (a) a single cell nucleus before DNA was released, (b) DNA released from a single nucleus in the absence of flow, (c) DNA extracted and stretched from a single nucleus by Method 1 and (d) the same, by Method 2

Fig. 5. Fluorescence micrographs of stretched DNA generated by Method 3. **(a)** Multiple bands of stretched DNA formed from a row of single nuclei. **(b)** Two bands of stretched DNA at a high magnification showing straight lines within the bands. **(c)** The full lengths of three bands of stretched DNA

Fig. 6. Fluorescence micrographs of (a) metaphase and (b) interphase telomere FISH of mouse embryonic stem cells. FISH signals are shown in green and chromosomal DNA, counterstained with DAPI, in blue. (c) Fluorescence micrograph of telomere FISH signals (indicated by arrowheads) in a band of chromosomal DNA (not counterstained) extracted and stretched from a single nucleus

Fig. 7.

The numbers of telomere fiber FISH signals plotted against their lengths