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Cell trapping, DNA extraction and Molecular Combing in a microfluidic device for high throughput genetic analysis of human DNA

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ABSTRACT

We report on an integrated microfluidic device for cell trapping, DNA extraction and Molecular Combing which can be used for genetic analysis of human DNA. Our results show that it is possible to isolate and linearize the genetic material of a few cells without introducing any manipulation step of DNA molecules, thus increasing dramatically the size of the events that can be studied on the genome. Such extraction strategy is simple and fast, providing a new possibility of high throughput single-cell DNA analysis which should be applicable to cancer research, genome mapping, and genotoxicity studies.

1. Introduction

Chromosomal aberrations consist of changes in the structure of DNA, such as rearrangements, deletions and/or amplifications, involving large portions of DNA molecules (up to the megabase scale). They may affect cell cycle regulation, differentiation, replication, apoptosis, and consequently cancer development [1]. In general, such events can only be visualized and studied with high molecular weight DNA molecules. In order to detect different types of chromosomal aberrations simultaneously at single-molecule level, DNA stretching techniques such as Molecular Combing (MC) can be applied [2]. However, the application of these techniques to small volume samples, like rare circulating tumor cells or micro-biopsies, is challenging because of the requirement of complete deproteinization of DNA molecules. Moreover, the size of the targetable genomic events is submitted to the fragmentation of genomic DNA molecules during manipulations. Human genomic (HG) DNA molecules have contour length in the mm range and are therefore easily fragmented when manipulated in solution and even more in microscopic environments [3]. On the other hand, microfluidic [4,5] and nanofluidic [6] devices previously designed could be used to elongate DNA molecules, but many other functionalities are not applicable to very large DNA molecules extracted from cells. Indeed, microchips integrating low molecular weight DNA extraction for PCR amplification have been successfully realized but they could not be combined with DNA stretching for large DNA analysis [7].

In this work, we report a novel microfluidic approach for *in situ* DNA extraction which is capable of generating arrays of megabase-long combed DNA from few tens of human cells. Our device was made in polydimethylsiloxane (PDMS) using standard soft-lithography techniques and reversibly bound to a vinyl-silane coated cover-slip. The microchip consisted of an array of micro-chambers connected to a main channel by small necks [8]. HeLa cells were introduced into the micro-chambers with a typical cell number of 0–15 cells per chamber, leaving an array of compartmentalized cellular material. This compartmentalization is maintained after removal of the PDMS device and digestion of both membranes and cellular proteins *in situ*. By modulating the adsorption of DNA molecules to the vinyl-silane coated surfaces, we were able to retain the genetic material with high efficiency and then perform MC. With this method, a high density array of long, stretched DNA molecules was constructed starting from few tens of cells. We observed stretched individual DNA molecules in the 100 μm/mm range, i.e., up to the size of a chromosome. The stretched DNA array is compatible with post-processing such as DNA hybridization of specific sequences and immunofluorescence detection of selected events on the genome. Moreover, the microfluidic approach opens up many possibilities and renders our method particularly versatile: by changing the configuration of the microchip, single cells could be targeted separately before extraction and testing conditions could be multiplexed in a single assay.

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2. Experimental procedures

2.1. Device fabrication

The microfluidic chip used in this work consisted of one layer of PDMS with microfluidic channels at the bottom bounded onto a vinylsilane-coated hydrophobic cover-glass. Photo-masks were drawn using L-Editor and printed on high resolution transparencies. Our final chip design (Fig. 1a) consisted of 70 circular micro-chambers of 60 or 70 μm radius, which were connected to a zig-zag main canal of 100 μm width with a neck of 20 μm width and 30 μm length. The neck sizes were chosen to allow both cell penetration (typical HeLa cell diameter in suspension = 10–15 μm) and fluidic stability in the micro-chambers. We tested different combinations of canal width (50, 75 and 100 μm) and neck sizes (15×20, 15×30, 20×30 and 30×40 μm) to render cell loading more reproducible. We found best results with the following ratios of canal width: neck width: neck length = 5: [1–1.5]: [1.5–2]. Larger canals allowed minimizing cell clogging during injection. The mold for the microfluidic channels was fabricated in a 50 μm-thick SU-8 3050 photoresist (Microchem Co., Ltd.) layer, spin-coated on a silicon wafer (Siltronix) at 3000 rpm prior to soft bake, UV exposure with a spot light source (LC5, Hamamatsu) through a transparency photo-mask, post bake, and development with SU-8 developer (Microchem) following the protocol described by the manufacturer. The SU-8 mold was rinsed with isopropanol, blown with a clean air flow, and coated with chlorotrimethylsilane (Sigma) for 3 min. To obtain the PDMS layer of the chip, liquid PDMS (RTV 615, GE; solutions A and B were mixed at the ratio of 10:1 (w/w)) was poured onto the mold to be ca. 5 mm thick, degassed and cured at 75 °C for more than 2 h. Afterward, the solidified PDMS layer was peeled off from the mold and the inlet and outlet holes were punched by using a syringe needle without pointed end (threaded hub needle gauge 21, Kahnetics). These holes were flushed with isopropanol and the device was cleaned with scotch tape (3 M) (Fig. 1b). The surface of the PDMS layer was rendered hydrophilic using an oxygen plasma cleaner (Harrick; 7 min, 200 mTorr, 18 W applied to RF coil) before being reversibly bounded on the silanised cover-glass (0.13–0.16 mm thick, 7-octenyl-thrichloro-silane coated, Genomic Vision) with the channel-fabricated surface down (Fig. 1c).

2.2. Cell culture and preparation

Cell trapping was performed using human cervical cancer cell line (HeLa cells) at P7. Cells were cultured to confluence in a standard T25 flask (Techno Plastic Products AG) at 37 °C, 5% CO_2. Growth Medium used was DMEM (Dulbecco's Modified Eagle Medium, Invitrogen) with 10% v/v FBS (Invitrogen). Cells were harvested with Trypsin–EDTA solution (Trypsin 0.05% in 0.53 mM EDTA, Invitrogen), counted, centrifuged at 1000 rpm for 5 min and resuspended in GM to a final concentration of 5×10^3 cells/μl.

2.3. Cell trapping in micro-chambers

Before injection of the cellular suspension, the chip mounted on the coverslip was placed in a vacuum chamber (100 mTorr) for 30 min. Thanks to PDMS high gas solubility, the degassing treatment generated a temporary pumping inside the microfluidic circuit as the material progressively reabsorbs air from the outside (Fig. 1c). The phenomenon obeys Henry's law, and PDMS 10:1 air absorption speed is reported to be larger than 4×10^{-5} cm/s [9]. To exploit the pumping and obtain spontaneous filling of the circuit, the experiment was thus performed within 20 min. The chip was positioned on a glass slide (1 mm thick, Menzel-Gläser) and two paper clips (3 cm large) were used to immobilize and compress the PDMS in order to avoid leakage and facilitate observation (Fig. 1d). Cell solution was mixed by vortex-pulse just before injection. 3 μL of sample solution (approx. 15,000 cells) were taken by micropipette and the tip was removed from the pipette. The tip was then gently tapped so that the sample solution was settled down to the tip end. Injection was performed by pushing the plastic tip inside the chip inlet and quickly removing it. The cellular solution progressively filled the array of chambers carrying cells inside them. Once all the micro-chambers were completely filled, the circuit was rinsed through the main channel with deionized water. After removal of the tubing, the chip was put at 37 °C for 3 h to let the remaining solution dry and facilitate cell adhesion on the chamber's bottom. Then the PDMS was gently removed from the coverslip.

2.4. DNA deproteinization and Molecular Combing

To allow the digestion of membranes and proteins, the coverslip was positioned on top of a 30 μL drop of digestion buffer (0.5 M EDTA pH 8 (Calbiochem), 1% *N*-lauroylsarcosine sodium salt (Sigma), 2 mg/ml Proteinase K (Eurobio)) on a clean glass slide and incubated for 8 h at 50 °C in a humid box. The surface was then recovered by gently immersing the slide in MES buffer pH 5 (Calbiochem). It was then immersed horizontally in a large reservoir filled with MES buffer pH 6 and incubated for 20 min before Molecular Combing. DNA stretching was performed using a MCS system (Genomic Vision), extracting the coverslip from the reservoir vertically at a constant speed of 300 μm/s.

2.5. Fluorescence microscopy

DNA molecules were stained with cyanine dye YOYO-1 iodide (Invitrogen). We used an Axio Observer inverted microscope (Zeiss)

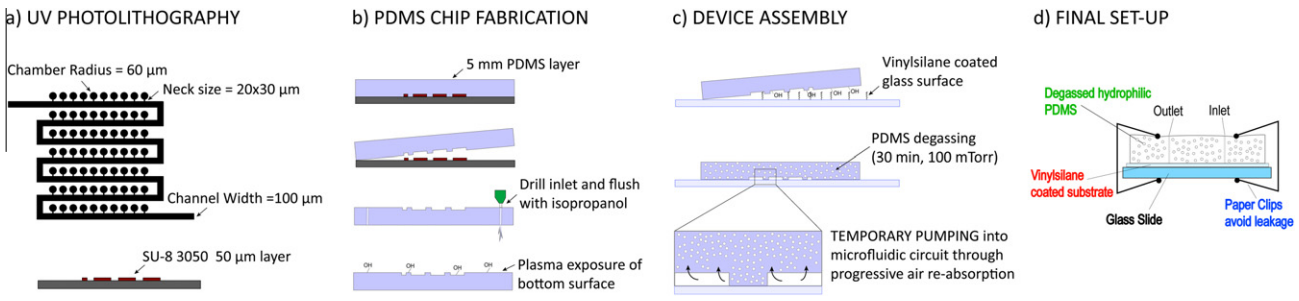

Fig. 1. Fabrication and preparation of the microfluidic chip. (a) Example of micro-chambers array layout and SU-8 mold fabrication. (b) PDMS layer fabrication and surface treatment. (c) Reversible assembly of the hydrophilic PDMS layer on the hydrophobic vinylsilane-coated surface: polymer degassing treatment helps PDMS-surface adhesion and creates a temporary pumping inside the circuit (d) Final device configuration: the degassed PDMS-surface device is punched on a glass slide with paper clips to avoid leakage during cells injection.

equipped with an EM-CCD camera, PhotonMAX (Princeton Instruments/Acton) and appropriate filters for YOYO-1 and AlexaFluor dyes. EC Plan-Neofluar 2.5×/0.075, N-Achroplan 5×/0.13, Plan-Apochromat 20×/0.8 were used to obtain images of this article. An HBO 100 W mercury lamp (Osram) was used as a light source.

3. Results and discussion

Fig. 2 shows our experimental procedure. Small groups of HeLa cells were meant to be trapped and immobilized on defined regions directly on the Molecular Combing glass substrate. As a consequence, our microfluidic device had to be reversibly bound to the coated surface, in order to recover the substrate after cell trapping. At the same time, the hydrophobic character of the vinyl-coated surface impeded low pressure loading of the cell solution inside the chip. To overcome this problem and meet the reversibility requirement we coupled hydrophilic treatment and degassing of the PDMS. The chip was put under vacuum for 30 min. and then punched on a glass slide (1.0 mm thick) with paper clips to avoid leakage (Fig. 1d). A pipette tip containing

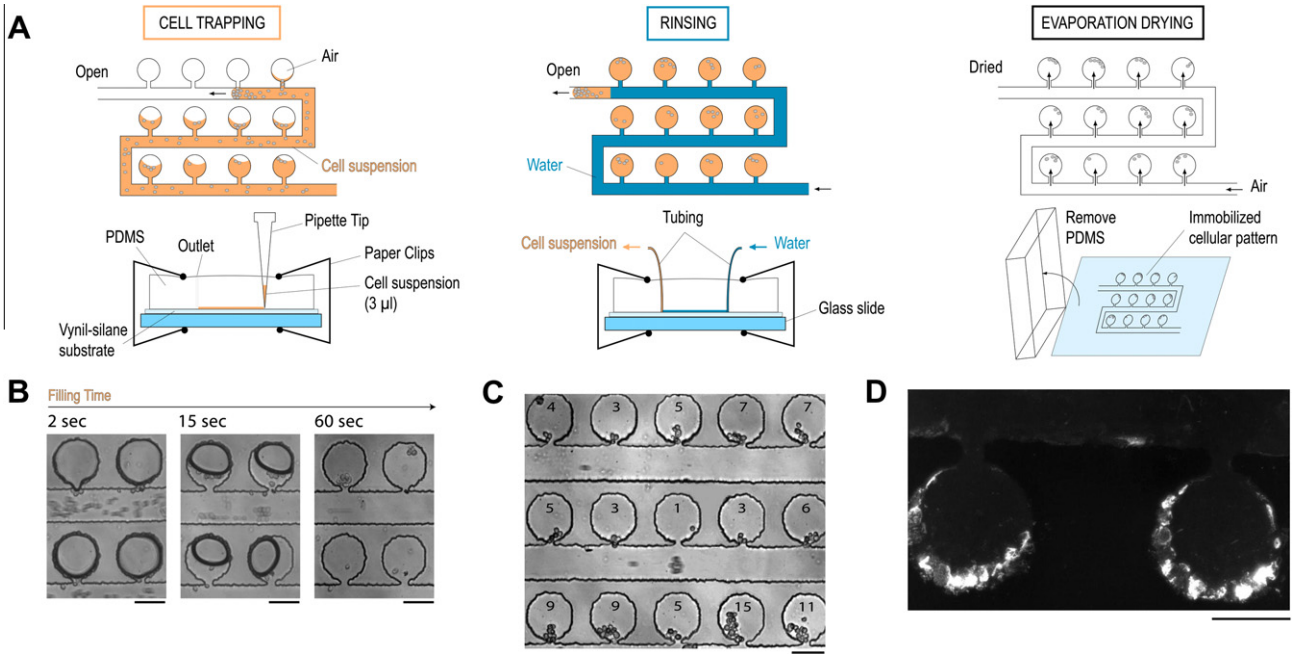

Fig. 2. Procedure to trap small groups of cells into the array of micro-chambers and immobilize the cellular material on the substrate. (A) Upper figures illustrate the microfluidic channel and lower figures show the chip configuration. (B) Time-lapse observation of the filling of the chambers by the cellular solution. (C) Optical micrograph showing small groups of HeLa cells irreversibly trapped into the chambers during rinsing. Numbers indicate amount of cells inside the corresponding micro-chamber. (D) Fluorescence image showing YOYO-1 stained DNA extracted from entrapped cells after removal of the microchip. Scale bars are 100 µm.

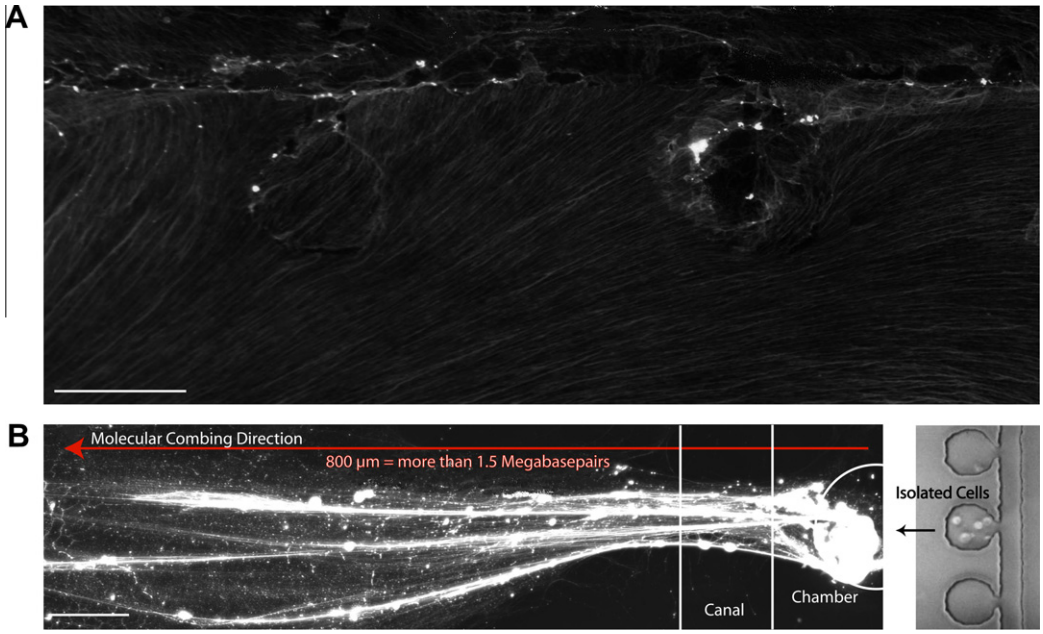

Fig. 3. Molecular Combing. (A) Fluorescence image showing the extracted DNA molecules of Fig. 2D after Molecular Combing. (B) DNA fibers extracted and combed from an isolated group of cells trapped in a single micro-chamber. Scale bars are 100 µm.

3 μl of cell suspension was inserted in the device inlet and quickly removed. A small plug of fluid was aspirated from the tip by the negative pressure present inside the PDMS and accelerated inside the main channel (Fig. 2A). As soon as the liquid reached a chamber's neck, an air bubble formed inside the chamber (Fig. 2B) and was progressively reabsorbed by the degassed PDMS. Differently from previously reported methods [9], we observed that cells were trapped inside chambers mainly by adsorption to the interface of such bubbles (Fig. 2B, right). This method proved really efficient, since less than 5000 cells were needed to trap more than 100 cells in approx. 1 min, with a cell distribution varying from 0 to 15 cells per chamber (Fig. 2C). Once the chambers filled, the outlet was connected to open tubing, and deionized water was introduced from the inlet. The cells were washed away by water in the main channel but remained in the chambers due to the high hydrodynamic resistance provided by the necks' design. Before removal of the PDMS layer, the cells were dehydrated inside the chip in order to preserve the compartmentalization. Then, DNA was extracted from the immobilized cells *in situ* by simple incubation of the cell-patterned coverglass with a proteolysis medium. The resulting array of adsorbed DNA stained with YOYO-1 appeared in the form of circular arcs (Fig. 2D), as the air interface had pushed the cellular material to the edge of the chambers during evaporation. The vinyl-silane coated surfaces efficiently bind DNA at acidic pH. By lowering the pH of the buffer, we were able to retain the genetic material with high efficiency and wash away the digestion residuals which could impede Molecular Combing. Increasing the pH to a value of 6 reduced the strong adsorption of DNA to the surface and allowed stretching the molecules to form a highly dense array (Fig. 3A). Molecular Combing was performed by pulling the substrate carrying the DNA out of the buffer solution at constant speed. Stretched molecules extended continuously from the chambers region over several millimeters, which correspond to millions of base pairs. Molecule alignment during combing was affected by the surface properties of the substrate. When cells were trapped in an isolated chamber (Fig. 3B), it was possible to achieve a better control of the stretching direction. However, in Fig. 3B DNA molecules appear entangled in microfibers, which is due to inefficient proteolysis. This aspect is currently under optimization in our laboratory.

4. Conclusion

Our results show that it is possible to isolate and linearize the genetic material of a few cells with good efficiency without introducing any manipulation step of DNA molecules, thus increasing dramatically the size of the events that can be studied on the genome. Such extraction strategy is simple and fast and opens up to the possibility of high-throughput single-cell DNA analysis, which is of great interest for cancer research, genotoxicity studies and genome mapping.

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