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Method Article

The method to dynamically screen and print single cells using microfluidics with pneumatic microvalves

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A B S T R A C T

Printing single cells into individual chambers is of critical importance for single-cell analysis using traditional equipment, for instance, single-cell clonal expansion or sequencing. The size of cells can usually be a reflection of their types, functions, and even cell cycle phases. Therefore, printing individual cells within the desired size range is of essential application potential in single-cell analysis. This paper presents a method for the development of a microfluidic chip integrating pneumatic microvalves to print single cells with appropriate size into standard well plates. The reported method provided essential guidelines for the fabrication of multi-layer microfluidic chips, control of the membrane deflection to screen cell size, and printing of single cells. In brief, this paper reports:

- the manufacturing of the chip using standard soft lithography;
- the protocol to dynamically screen both the lower and the upper size limit of cells passing through the valves by deflection of the valve membrane;
- the screening and dispensing of suspended human umbilical vein endothelial cells (HUVECs) into 384-well plates with high viability.

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A R T I C L E I N F O

Method name: Dual pneumatic microvalves method for single cell screening*Keywords:* Dynamic screening, Printing, Microfluidics, Pneumatic microvalves, Single cells

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Specifications table

Subject Area:	Engineering
More specific subject area:	Single-cell screening
Method name:	Dual pneumatic microvalves method for single cell screening
Name and reference of original method:	C. Chen, D. Xu, S. Bai, Z. Yu, Y. Zhu, X. Xing and H. Chen, <i>Dynamic screening and printing of single cells using a microfluidic chip with dual microvalves. Lab on a Chip</i> , 2020. 20(7): p. 1227–1237.
Resource availability:	/

Background

Isolating and inoculating individual cells into well plates for subsequent analysis has emerged as an important research direction for high-throughput single-cell studies [1]. As an ascendant technology for cell study, microfluidic technologies have been exploited for not only on-chip analysis such as cell culture [2,3] and biomarker analysis [4], but also pre-treatment on chip before further analysis using traditional equipment, e.g., cell inoculation and CRISPR-Cas9 delivery [5]. Inkjet-like printing [6] has achieved automatic and high-speed isolation of single cells by combining cell manipulation technologies, such as vacuum system [7,8], acoustophoretic focusing [9], and impedance-based cell detection [7,8]. This technique selects the droplet containing one cell and flying outside the chip using an image visualization system. Accurate dispensing techniques with size selection has also been explored for single-cell inoculation [10]. They can control the lower size limit of cells using a fixed micropillar array [10]. Trap-release structures [11] have the same size selection capability but can dynamically screen cell size by a single-layer pneumatic microvalve. To achieve the dynamic control of both the upper and lower limit of the cells for printing, a microfluidic chip integrated with dual pneumatic microvalves was developed by Chen et al. [1]. The human umbilical vein endothelial cells (HUVECs) were employed to demonstrate the dynamic screening capability of the technique. The cell viability test suggested that the screening and printing process had no significant damage to cells.

This paper introduces the details of the fabrication of the multi-layer microfluidic chip, control of the membrane deflection to screen cell size, and printing of single cells. It aims to provide essential information for the development of microfluidic chips with pneumatic valves and single-cell manipulation.

Design of the microfluidic chip

The planar design of the chip was depicted in Fig. 1A. The chip has three layers: the gas layer, the membrane layer, and the fluid layer. The gas channels (blue ones in Fig. 1A) are 100 μm (width) \times 25 μm (height). The flow channels consist of an inlet, a printing outlet, and a waste outlet. In the fluid channel, the pre-screening unit was a multilevel microfilter structure with openings in the range of 200 to 25 μm . All channels following the pre-screening unit were 25 μm wide. The 10 μm membrane was sandwiched between the two layers (Fig. 1B) and the valve region was formed at the intersection of two channels. The microfluidic network layouts were created using AutoCAD2018 (Educational version) and attached as a supporting document.

Device manufacture

The masters of the flow and pneumatic layers were manufactured by standard photolithography in the clean room [3,12]. After 30 min of dehydration at 100 $^{\circ}\text{C}$, a 25 μm thick film was then created on the silicon wafer by spin-coating the SU-8 2025 negative photoresist (MicroChem, Newton, MA, USA) at 3570 rpm for 30 s. The coated wafer was then soft-baked for 5 min at 95 $^{\circ}\text{C}$ on a hot plate within a fume hood. The SU-8 film was then exposed to UV light (URE-2000, Chinese Academy of Sciences, Beijing, China) with the energy of 150 mJ/cm^2 under a chrome mask, before baking at 65 $^{\circ}\text{C}$ for 1 min

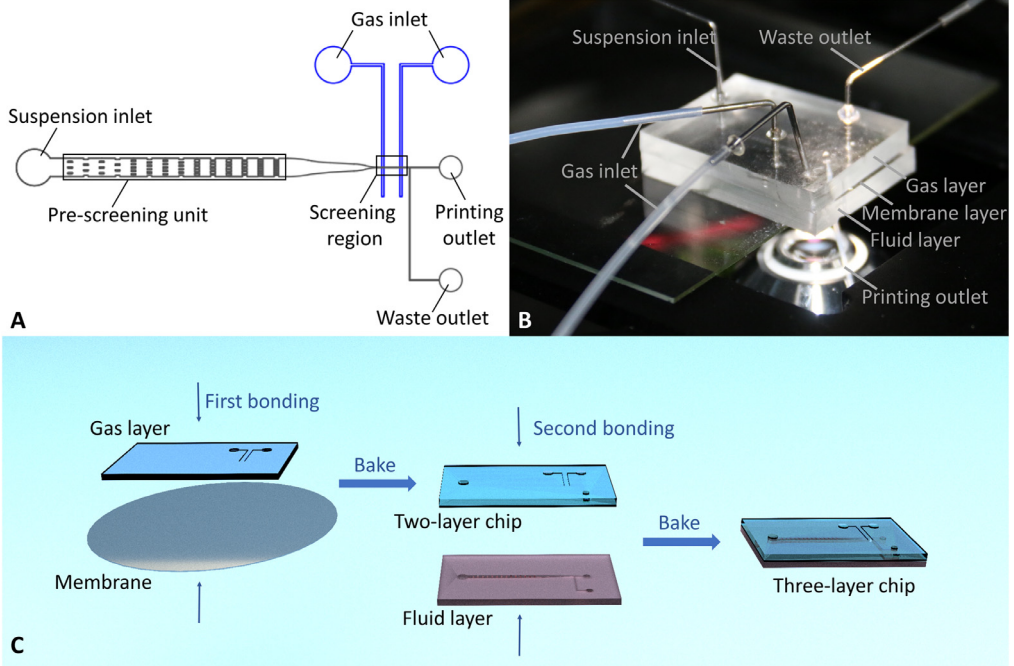


Fig. 1. The microfluidic chip with dual microvalves for single-cell screening and printing. A) Top view of the design for an assembled chip. The blue and black channels are the gas and fluid channels, respectively. B) The photograph of the microfluidic chip on a microscope stage. C) The schematic drawing of the manufacturing processes for the multiple-layer chip.

and 95 °C for 5 min. Afterward, the SU-8 film was developed in the SU-8 developer (MicroChem) for 4 min. Finally, the master was rinsed using acetone and IPA, and dried using the compressed nitrogen.

The flow and pneumatic layers were fabricated by casting uncured PDMS against the masters [12,13]. After standard photolithography, the SU-8 masters were exposed to the vapor of Trichloro (1H,1H,2H,2H-perfluorooctyl)-silane (Aladdin®, Shanghai, China) for 15 min to facilitate the following demolding. Sylgard 184 elastomer base and curing agent (PDMS, Dow Corning® Corporation, Midland, USA) were mixed for 20 min with a weight ratio of 10:1 and casted to the SU-8 masters, before complete degassing. After 3-h baking in an oven at 60 °C, the cured PDMS replicas were peeled off from the masters.

The PDMS membrane was spin-coated on the silicon wafer. The membrane thickness at various spin speed was reported elsewhere [1,3]. When the spin speed increased from 1500 rpm to 5500 rpm and spin time maintained at 90 s, it is possible to manufacture membranes with thickness ranging from 43 to 10 μm. This relationship can be described by [1]:

$$t = 1.439 \times 10^{-6}v^2 - 0.01811v + 67.39.$$

Where t (μm) is the thickness of the membrane and v (rpm) is the spin speed.

The proportion of curing agent and silicone base was maintained at 1:25 to obtain a less rigid membrane with Young's modulus of 0.98 MPa [14]. After the 15-minute salinization of the silicon wafer using Trichloro (1H,1H,2H,2H-perfluorooctyl)-silane, the degassed PDMS mixture was spin-coated on the wafer at 5500 rpm for 90 s. Afterward, the wafer was placed in an oven at 60 °C for 3 h to completely cure the PDMS film.

Three PDMS layers were irreversibly bonded together (See Fig. 1C) following oxygen plasma oxidization. Firstly, the through holes for the gas inlets and the printing outlet were punched on both

the gas and the fluid layers using a handheld punch (WH-CF-13, I.D. = 0.5 mm, Wenhao, Suzhou, China). Then, the gas layer and the membrane attaching to the wafer were treated by oxygen plasma for 30 s using a plasma cleaner (PDC-002, Harrick Plasma, USA), before they were brought into conformal contact to form irreversible bonding. After baking for three hours in an oven at 60 °C, both layers were peeled off from the wafer. The through holes for the inlet and the waste outlet were punched using methods mentioned above. Finally, the fluid layer was irreversibly bonded to the membrane on the gas layer using the aforementioned protocol.

Platform construction

The microfluidic chip was connected to airtight tubes via tubing with the inner and out diameter of 0.5 and 1.1 mm, respectively. A pressure controller (ELVEFLOW® OB1 Mk3) was used to precisely regulate the pressure (both negative and positive) to either activate the pneumatic microvalves or drive the fluid flow for printing. Two gas channels were supplied with the pressure ranging from 0 to 2000 mbar to control the microvalves. The cell suspension was stored in a tube with an airtight cap and driven into the inlet of the chip by 200 mbar channel on the pressure controller. The positive pressure inside the channel network efficiently prevented the formation of the air bubbles. The waste outlet was connected with an airtight tube for waste cell collection using the negative pressure channel (ranges from -900 to 1000 mbar) on the pump. The chip was placed on a glass slide with through holes allowing the connection of the tubing to the well plate from the bottom of the whole chip. Finally, the microfluidic chip was mounted on an inverted microscope (IX83 Olympus, Japan) equipped with a CCD camera (C11440-36U, Hamamatsu, Japan) to image the microchannels.

Membrane deflection prediction and control

The operation protocols to screen and print cells with desired sizes were based on channel clearance defined by the deflected valve membrane. The valve membrane was precisely regulated by the positive pressure from the pressure controller. The pressure applied on both microvalves to screen cells was determined according to the empirical equation below [1]:

$$P = 2.12 - 0.096D_{\text{cell}}$$

Where P (atm) was the applied pressure and D_{cell} (μm) was either the upper or the lower diameter limit of the cell for screening.

Cell screening and printing

The microfluidic channels and tubing were sterilized by perfusing 70% ethanol (Ante, Anhui, China) at 1 $\mu\text{L}/\text{min}$ for 30 min. The low surface tension of the ethanol could help the fill-up of the microchannels without the formation of bubbles. Afterward, the channels were rinsed completely by injecting sterilized DI water at 1 $\mu\text{L}/\text{min}$ for 10 min. Finally, the inner surface of microchannels was primed by 1% Pluronic (Saen, Shanghai, China) solution injected at 1 $\mu\text{L}/\text{min}$ for 30 min to prevent cell adhesion.

The dual pneumatic microvalves were designed for screening suspended cells. The front microvalve activated using a bigger pressure (P_1) determined the lower limit (d_1) of the cells, whereas the back one applied with a smaller pressure (P_2) adjusted the upper limit (d_2) of the cells [1]. During the cell screening experiments, the microvalves were controlled manually using the supporting software called Elveflow® Smart Interface.

Finally, HUVECs were employed as a model to demonstrate the screening and printing capability of the microfluidic chip. After trypsinizing and resuspending of HUVECs in the medium, the cell suspension was stored in the airtight tube (Fig. 2) for cell printing for less than 1.5 h to avoid the influence of the cell viability by suspension state. After deforming the microvalves, a pressure of approximately 200 Pa was applied to the tube with cell suspension to drive cells to flow into the microfluidic chip. The driving pressure can be fine-tuned according to the flow speed

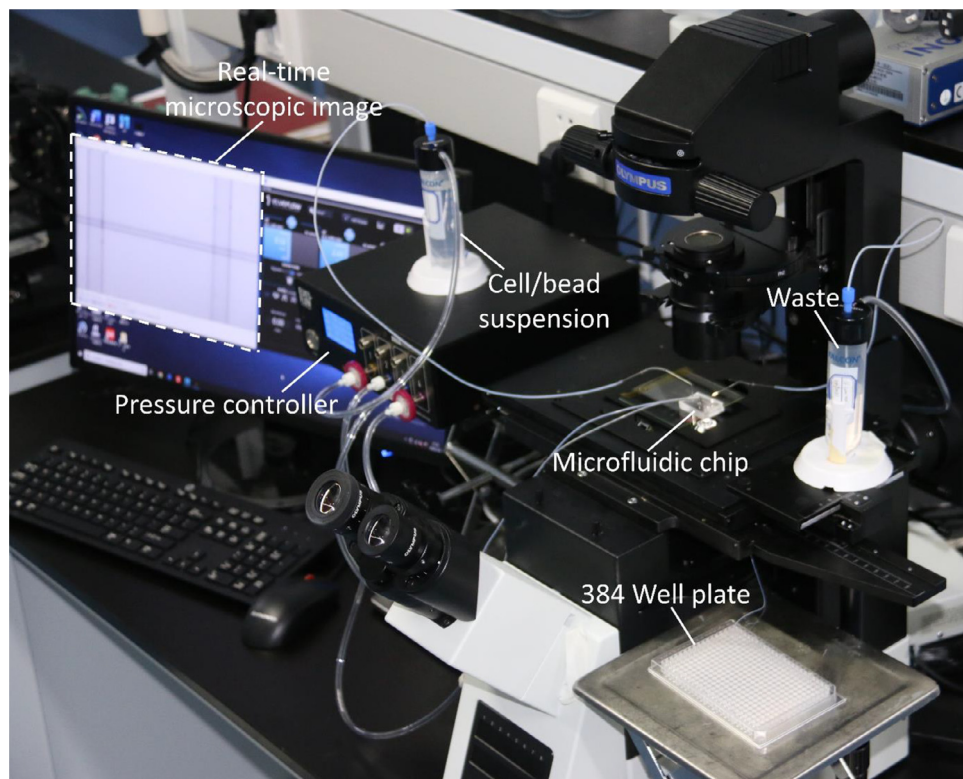


Fig. 2. The experimental platform for dynamic screening and printing of single cells/beads.

of the cells but small pressure is preferable to avoid remarkable cell deformation under the microvalves [15].

The following protocol demonstrates the printing of cells with a size between d_1 and d_2 . If the cell passes through the first valve directly, it suggests the cell is smaller than d_1 and a 100 Pa negative pressure will be applied to the waste outlet to recover the cell (see the left route in Fig. 3). On the other hand, if the cell is trapped by the first valve, it indicates the cell diameter is bigger than d_1 and the pressure (P_1) on the front valve will be released to fully open it.

After the opening of the front valve, the following operation is determined based on the cell motion through the rear valve. If the cell passes through the second valve directly, it suggests the cell is within the desired size range ($d_1 < d < d_2$) and a small positive pressure (smaller than inlet pressure) will be applied to the waste outlet to drive the cell to the printing outlet and then the well plate (see the middle route in Fig. 3). On the contrary, if the cell is trapped by the rear valve, it indicates the size is larger than the desired upper limit (d_2). As a result, a small negative pressure (about 100 Pa) will be applied to the waste outlet to draw out the cell after the opening of the rear valve (see Fig. 3 right route).

It is possible to acquire cells with sizes smaller than d_1 or larger than d_2 just by controlling the pressure in the waste channel to direct the flow direction of the cells. Moreover, the deflection of valves can be flexibly adjusted by the positive pressure, thus the upper or lower limit of the size of the cell can be dynamically altered during the same experiment. Therefore, the microfluidic chip with dual valves is capable of dynamic size selection and printing of single cells. It will be of broad application potential in the field of single-cell study.

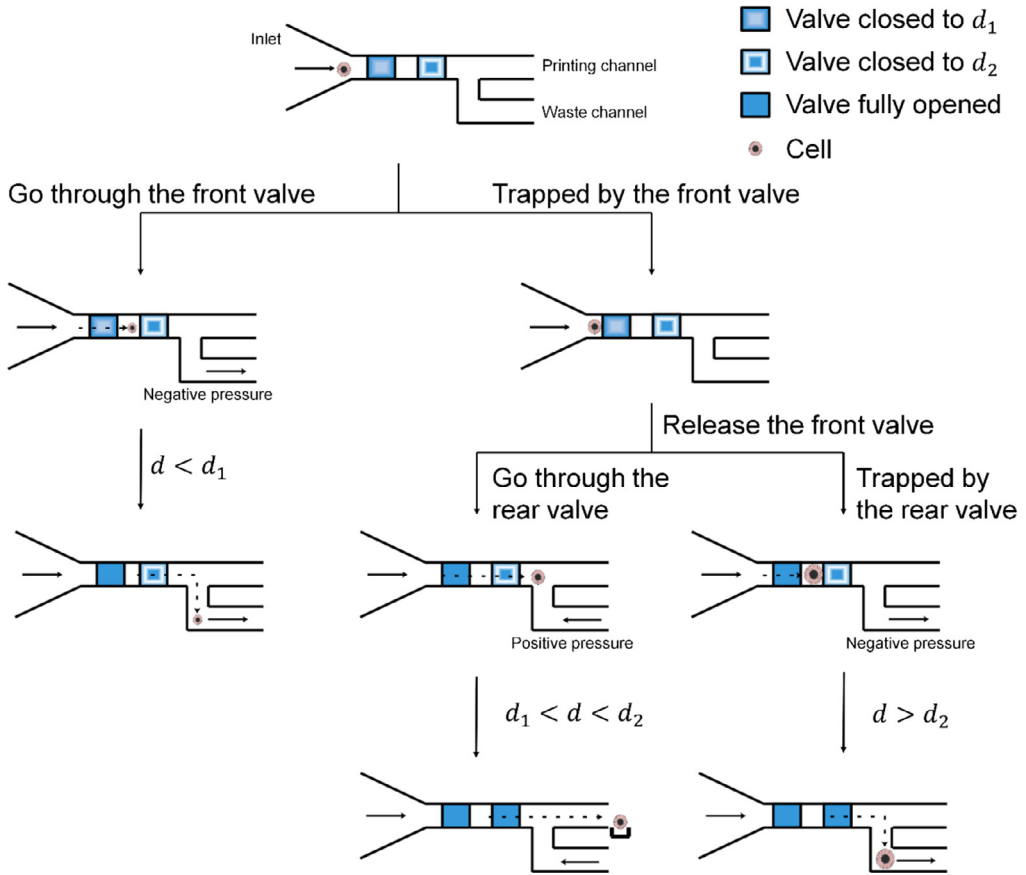


Fig. 3. The schematic diagrams of the screening and printing of single cells with desired sizes in the microfluidic channels with two microvalves.

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Declaration of Competing Interests

The Authors confirm that there are no conflicts of interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi: 10.1016/j.mex.2020.101190](https://doi.org/10.1016/j.mex.2020.101190).

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