Single-cell isolation by a modular single-cell pipette for RNA-sequencing†

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Single-cell transcriptome sequencing highly requires a convenient and reliable method to rapidly isolate a live cell into a specific container such as a PCR tube. Here, we report a modular single-cell pipette (mSCP) consisting of three modular components, a SCP-Tip, an air-displacement pipette (ADP), and ADP-Tips, that can be easily assembled, disassembled, and reassembled. By assembling the SCP-Tip containing a hydrodynamic trap, the mSCP can isolate single cells from 5–10 cells per μL of cell suspension. The mSCP is compatible with microscopic identification of captured single cells to finally achieve 100% single-cell isolation efficiency. The isolated live single cells are in submicroliter volumes and well suitable for single-cell PCR analysis and RNA-sequencing. The mSCP possesses merits of convenience, rapidness, and high efficiency, making it a powerful tool to isolate single cells for transcriptome analysis.

Introduction

Single-cell RNA-sequencing (RNA-seq) is becoming a strong molecular biology tool and accelerating the understanding of how individual cells differ from others and respond to perturbations.1–4 The primary step for successful single-cell RNA-seq highly requires a convenient and reliable method to rapidly isolate a live cell into a submicroliter suspension and then transfer it into a specific container such as a PCR tube for genetic analysis.5–7 However, it is still challenging for micromanipulation8–11 and fluorescence activated cell sorting (FACSs),12,13 the two most common ways for single-cell isolation, to completely satisfy the above requirements. In the micromanipulation, single cells are usually aspirated into a glass capillary (typically 30 μm in diameter) by applying a gentle negative pressure which can be provided by a manual/automated micropipettor (called micro-pipetting) or even a researcher’s mouth (called mouth-pipetting). It relies much on personal skills because the key process of single-cell aspiration should be carefully completed under a microscopic field. Although relatively accurate, it is time consuming and has a low throughput.5 FACS, in contrast, is a fast and automated method for single-cell isolation. However, cell viability and integrity may be affected by the high shear force from the sheath fluid. Additionally, at least several thousands of input cells are required, making it unable to achieve effective single-cell isolation from a small number of cells.

Recently developed microfabrication-based techniques, especially microfluidics-based methods, provide powerful platforms for high-efficiency or high-throughput single-cell isolation by the combination of specially designed microstructures with precise manipulation of microfluids.14–16 According to the difference of container for single-cell isolation, these platforms can be divided into three types: 1) a microtrap-based platform17–19 such as C1 Single-Cell Auto Prep IFC (Fluidigm) where single cells are fluidly captured by hydrodynamic traps and isolated from the surroundings by closing the valves; 2) a microdroplet-based platform20,21 such as the Chromium Single Cell 3’ Solution (10× Genomics) where both single cells and barcoded beads are simultaneously encapsulated into rapidly flowing nanoliter-sized aqueous droplets; and 3) a microwell-based platform22,23 such as the ICELL8 Single-Cell System (WaferGen) where single cells are randomly trapped into round microscale wells and subsequently confirmed by a microscope. These platforms integrate the functions of single-cell isolation and molecular amplification; however, they are either difficult to operate (self-made) or to access (commercial) due to the requirement of expensive instruments (several hundred thousand dollars). These make the implementation of single-cell study in common biological

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c6lc01241h
laboratories largely restricted, where single cells are regularly captured and transferred into PCR tubes, followed by the lysis and amplification of minute amounts of mRNA from the isolated single cell.

We reported a single-cell pipette (SCP), allowing for rapid single-cell isolation from cell suspensions. The SCP is a handheld system with great potential. The current SCP system still requires a self-made pressure generator made of two 1 mL syringes to generate the working pressure empirically and is limited by a relatively high cell concentration (≥10^3 cells per μL). Here, we report a modular SCP (mSCP) which overcomes the above limitations. (1) Pressures are provided by a common air-displacement pipette (ADP), allowing for more convenient operation and gentle pressure control. (2) By combining with microscopic identification, the mSCP can achieve 100% efficiency in single-cell isolation. (3) By equip-ping with a SCP-Tip containing a hydrodynamic trap, the mSCP enables isolation of single cells from a relatively low concentration of cell suspension. With the new capability, we achieved single-cell isolation by the mSCP from 5–10 cells per μL of cell suspension.

Materials and methods

Design and fabrication of SCP-Tips

The SCP-Tips, including the SCP-Tip containing a hook and the SCP-Tip containing a hydrodynamic trap, were designed using AutoCAD software (Autodesk) and fabricated by photolithography and polydimethylsiloxane (PDMS) molding techniques. In brief, the design was printed out as five-inch glass photomasks (Photo Sciences, Inc.) and then transferred to the surface of a four-inch silicon wafer as an 18 μm thick SU-8 3025 negative photoresist (MicroChem Corp.). After silanization by trimethylchlorosilane (TMCS), polydimethylsiloxane (PDMS; 10A: 1B; Dow Corning Corp.) was poured onto the photoresist mold, degassed by vacuum for 15 min, and heated at 80 °C for 25 min. After curing, the PDMS was peeled off and two tilted holes were punched by a puncher (Harris Uni-Core). Then, the upper PDMS layer (4–5 mm thickness) without a microstructure was irreversibly bonded to the bottom PDMS layer (0.5–1 mm thickness) without a microstructure by plasma treatment (Plasma ETCH, INC) to form an intact chip. The chip was left at 80 °C for 30 min to enhance the bonding. Finally, the chip was cut to the appropriate size and shaped by using a scalpel to finally form a SCP-Tip.

Preparation of cells

The cell lines MDA-MB-231/GFP (Cell Biolabs) and NIH 3T3 (ATCC) were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% penicillin–streptomycin. K562 cells (ATCC) were cultured in Iscove’s modified Dulbecco’s medium, supplemented with 10% (v/v) FBS and 1% penicillin–streptomycin. All cells were grown in a humidified atmosphere of 5% (v/v) CO2 at 37 °C. Adherent cells (MDA-MB-231 and NIH 3T3) at 80% confluence in a 60 mm × 15 mm Petri dish were harvested by trypsin digestion and dispensed into a cell suspension with various concentrations according to the experimental requirement. Suspended cells (NK-92 and K562) were centrifuged and washed with cell-free media or PBS and finally adjusted to known cell concentrations.

Detailed assembly of mSCP

The detailed assembly of the mSCP is as follows. (1) Insert an ADP-Tip (20–300 μL, #022491547, Eppendorf) filled with 50–100 μL of cell-free liquids into the bottom of the +P port of the SCP-Tip. (2) Use the middle finger to slightly flick the ADP-Tip to remove all air bubbles. (3) Insert an empty ADP-Tip (20–300 μL, #022491547, Eppendorf) into the bottom of the −P port of the SCP-Tip. (4) Connect the ADP (20–200 μL, ES-200, Eppendorf) to the +P ADP-Tip and apply a +P (200 μL) for around 20 seconds to replace air with the liquid. (5) Disconnect the ADP with the +P ADP-Tip and immerse the end of the SCP-Tip into a small container filled with liquid to prevent potential clogging of the microchannel at the SCP-Tip end due to liquid evaporation.

Detailed operation of mSCP for single-cell isolation

The detailed operation of the mSCP for single-cell isolation is as follows. (1) Dispense one 5–20 μL cell suspension and three 40–50 μL cell-free liquids into four small containers, respectively. (2) Re-suspend cells uniformly before each use by a pipette to prevent cell sedimentation. (3) Push the piston button down of the ADP and connect to the −P ADP tip. (4) Completely release the piston button to generate a −P (~200 μL for the mSCP-Tip containing a hook and ~50 μL for the mSCP-Tip containing a hydrodynamic trap) and quickly immerse the SCP-Tip end into the uniform cell suspension for 10–20 s. (5) Wash residual cells by moving the SCP-Tip end up and down several times into cell-free liquids consecutively. (6) Disconnect the ADP from the −P ADP-Tip and put the SCP-Tip on a microscope stage for single-cell identification by a ×10 objective lens. (7) Connect the ADP to the +P ADP-Tip and push the piston button down to generate a +P of 200 μL for 5–10 s to release the identified single cell to the SCP-Tip end and transfer the single-cell droplet into a desired container. (8) Disconnect the ADP from the +P ADP-Tip while pushing the piston button down to avoid the liquid from going back. (9) Repeat operations of (2)–(8) to pick up more single cells.

Single-cell droplet volume evaluation

After the microscopic identification, the single cell was released to the SCP-Tip end and transferred onto a Petri dish.
RNA-seq and data analysis

After single-cell isolation by the mSCP, cDNA synthesis and amplification (SMART-Seq v4 Ultra Low Input RNA Kit), cDNA purification (Agencourt AMPure XP Kit), and cDNA library synthesis (Nextera XT DNA Library Prep Kit) were implemented according to the manufacturer’s instructions. All libraries were sequenced on a HiSeq 2500 (Illumina) platform. 100 bp paired end short reads were generated for subsequent bioinformatics analysis. The bulk and single cell RNA-seq paired-end reads were mapped to a human reference genome (hg19) using Tophat. The quality of the reads was estimated using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and the numbers of transcripts with FPKM (fragments per kilobase of exon per million reads) were calculated from the cufflinks and CummeRbund (http://compbio.mit.edu/cummeRbund/) pipelines. A scaling scatter plot was created to show the relationship between all pairs of genes using the R “ggplot2” package.

Acquisition of images and movies

Bright-field and fluorescence images were obtained using an EVOS FL imaging system and a T3i Canon camera. Movies were filmed by the EVOS FL Auto imaging system and the T3i Canon camera.

Results and discussion

The mSCP consists of three modular components: SCP-Tip, ADP-Tips (−P ADP-Tip and +P ADP-Tip), and ADP (Fig. 1). In particular, both ADP and ADP-Tips are commercially available. Therefore, these modular components can be easily assembled, disassembled, and reassembled. The key part of the mSCP is the SCP-Tip mainly containing a Y-bifurcation microchannel for fluid pressure transmission and a microscale hook within the straight microchannel for single-cell capture (Fig. 1A and Fig. S1–S2†). The SCP-Tip was designed using AutoCAD and fabricated by photolithography and polydimethylsiloxane (PDMS) molding techniques. On the top surface of the SCP-Tip, two 60 degree ports (−P port and +P port with 1.5 mm diameter and 5 mm length) were created for the convenient insertion of two commercially available ADP-Tips (Fig. 1B, upper left). Specifically, an empty ADP-Tip is inserted into the −P port as the −P ADP-Tip and an ADP-Tip filled with 100 μL of cell-free liquids, such as cell culture media or phosphate-buffered saline (PBS), is inserted into the +P port as the +P ADP-Tip (Fig. 1B bottom right). The maximum outside diameter of the inserted ADP-Tips (2 mm) is a little larger than the diameter of the pressure port (1.5 mm). Therefore, due to the excellent elasticity of PDMS, interfaces between the SCP-Tip and ADP-Tips are well sealed without leakage of gasses and liquids. By connecting the ADP with different ADP-Tips, the gentle pressure control, including negative pressure (−P) for single-cell capture and positive pressure (+P) for single-cell release, can be respectively achieved (Fig. 1C). In detail, only −P will be applied by connecting the ADP with the −P ADP-Tip (empty) and only +P will be applied by connecting the ADP with the +P ADP-Tip (containing the cell-free liquid). After completion of assembly of the mSCP, the gas in the Y-bifurcation microchannel is easily replaced with the cell-free liquid by applying 200 μL volume of +P. As a result, a small volume of liquid will flow out and form a submicroliter droplet at the end of the SCP-Tip.

Single-cell isolation can be achieved in 3 steps (Movie S1†).

Step 1: single-cell capture and washing. After applying −P to
the –P ADP-Tip by the ADP, the SCP-Tip end is rapidly immersed into a cell suspension for 10–20 seconds to allow cells to be aspirated into the straight microchannel (Fig. 2A). During this process, one cell will be randomly captured by the hook. The mSCP can also combine with well-established cell enrichment methods, such as FACS and magnetic separation, for improved target single cell isolation. According to the fluid distribution in the Y-bifurcation microchannel, cells only flow into the –P port and no cells will flow into the +P port (Fig. 1C, upper). The residual cells at the SCP-Tip end are washed out by dipping the SCP-Tip end into cell-free liquids while moving up and down several times (Fig. 2A). Meanwhile, under the effect of –P, all the uncaptured cells within the straight microchannel will be rapidly aspirated into the –P port and the –P SCP-Tip.

In order to obtain the optimum of pressure control for single-cell capture, three types of –P were investigated, including –100 μL, –150 μL, and –200 μL (Fig. 2B). That’s because the ADP compatible with the ADP-Tips has 20–200 μL in the volume range. The liquid velocity in the straight microchannel was calculated by measuring it in the microtubing (Fig. S3†). Results showed that the average liquid velocity within 10 seconds increased linearly with the applied –P, such as 30 mm s⁻¹ under the –P of 200 μL. Correspondingly, the average cell velocity evaluated through a time-lapse video was 22 mm s⁻¹, a little smaller than the average liquid velocity. The higher –P means more cells can be aspirated into the SCP-Tip and the hook has more possibility to successfully capture a single cell. Moreover, under the –P of 200 μL, the morphology of captured single cells could still be well

![Fig. 2](image-url)
maintained, such as MDA-MB-231 (breast cancer cells) with a diameter of 12–20 μm, NIH 3T3 (fibroblasts) with a diameter of 10–18 μm, and NK-92 (natural killer cells) with a diameter of 9–24 μm (Fig. S4†). Only one cell was captured at a time. Therefore, the –P of 200 μL provided by the ADP was selected. As a pressure generator in the mSCP, the ADP has three obvious advantages. (1) Widely available: the ADP is commercially available and has been the most common laboratory tool for liquid handling. (2) User-friendly: one can grasp the operation in the shortest time. (3) Gently controllable: both the –P and the +P can be gently provided just by adjusting the range of volume.

Step 2: single-cell identification. Due to easy disassembly between the ADP and the ADP-Tips, then the combined Tips (SCP-Tip and ADP-Tips) are separated from the ADP and put on a Petri dish for microscopic identification. With the merit of good optical transparency of PDMS30 and flat bottom of the PDMS SCP-Tip, the captured single cell by the hook can be easily and clearly identified under a common inverted microscope (Fig. 2C). Step 3: single-cell release and transfer. Due to poor cell adhesion on the PDMS surface,31 the captured single cells can be easily released by applying +P. In order to allow the captured single cells to be flowed out within the shortest time, the +P of 200 μL (maximum volume range) is applied to the +P ADP-Tip for 5–10 seconds to release the identified single cells to the SCP-Tip end and finally transferred into a container (Fig. 2D).

To achieve successful single-cell RNA-seq, three conditions are required for the sample preparation, including the guarantee of isolation of only one cell in each container, less than 1 μL cell suspension volume, and sufficient cell viability.32 The mSCP can meet all the above requirements. (1) One cell in one container. The average single-cell isolation efficiency by the mSCP was proportional to the cell concentration, such as 93.3% in 10^5 cells per μL and 86.7% in 10^3 cells per μL (Fig. 2E). Due to the hydrodynamic effect around the hook,24,29 the failure frequency increased significantly with the decrease of cell concentration, such as 3.3% in 10^2 cells per μL. However, by adding the step of microscopic identification (step 2), the mSCP could achieve 100% single-cell isolation (20 experiments), in which only the single cells identified by the microscope were released to the SCP-Tip end and then transferred as single-cell suspensions. The identification process was easy and rapid (≤20 s per cell). (2) Submicroliter single-cell suspension. Due to the smaller cross-sectional area of the straight microchannel (43 μm in width and 18 μm in height), the extruded liquid by the +P of 200 μL is in submicroliter volume with an average value of 500 nL (Fig. 2F and Movie S1†). After isolation of single cells into PCR tubes, in order to avoid potential cell damage from liquid evaporation, a cell lysis buffer will be rapidly added within 30 seconds after single-cell isolation. (3) High cell viability. Single cells are not damaged during the relatively short operational time (≤30 s) and low flow velocity (30 mm s⁻¹). Therefore, the cell morphology was well maintained after transfer (Fig. 2D). To further evaluate cell viability, 20 MDA-MB-231 cells were isolated by the mSCP into wells of a 96-well plate and 15 of them finally generated clones after 5 days of culture (Fig. 2G–H). As a comparison, 21 out of 25 single cells could generate clones by the method of serial dilution. There was no significant difference for them to generate clones.

As a proof-of-concept experiment, two MDA-MB-231 cells, named single-cell 1 and single-cell 2, were isolated into two PCR tubes by the mSCP, respectively, followed by cDNA synthesis, amplification, purification, library synthesis, and RNA-seq. The bulk RNA was obtained from a group of bulk cells (~1000 cells) and applied as a benchmark for comparison. Analysis results (Fig. 3) showed that correlations between single cells and bulk cells in RNA-seq measurement of gene expression were quite high with Pearson correlation coefficients of 0.973 (single-cell 1 vs. single-cell 2), 0.886 (single-cell 1 vs. bulk cells), and 0.884 (single-cell 2 vs. bulk cells), respectively, which were well consistent with a literature report.33

Moreover, the SCP-Tip containing a hydrodynamic trap34 was also developed to meet the requirement of single-cell isolation from a relatively low concentration of cell suspension (Fig. 4A and Fig. S5†). The trap was compatible with various cells, such as NIH 3T3 with a diameter of 13–18 μm, K562 (lymphoblasts) with a diameter of 12–17 μm, and NK-92 with a diameter of 11–20 μm (Fig. S6†). The fluid resistance in the microchannel is a little higher than that in the trap, so a single cell prefers to flow into the trap (Fig. 4B). As a result,
the single cell will be certainly captured by the trap once it is aspirated into the microchannel by applying \(-P\) to the \(-P\) ADP-Tip (Movie S2†).

The work flow of the SCP-Tip containing a trap for single-cell isolation is similar to the operation of the SCP-Tip containing a hook (Fig. 4C). The main difference is that the applied \(-P\) (−50 μL) is smaller and the aspiration time is longer (20 s). As expected, the average liquid velocity increased with the applied \(-P\) (Fig. 4D). Although under a high \(-P\) (≥−75 μL) single cells have more opportunities to be aspirated into the microchannels, the captured single cell was easy to deform, greatly increasing the possibility of escaping from the trap (Fig. 4E). Therefore, the \(-P\) of 50 μL provided by the ADP was selected. Under the effect of such a low \(-P\), the morphology of captured single cells could still be well maintained (Fig. S6†), indicating good cell integrity and high cell viability. In this process, the ADP plays a key role by providing gentle pressure control. The average single-cell isolation efficiency was proportional to the cell concentration, such as 96.7% in 10^2 cells per μL and 76.7% in 10 cells per μL (Fig. 4F). That’s because cells are not always uniformly dispersed in the solution and sometimes no cell is aspirated into the microchannel. And the probability of failure increased with further decrease of cell concentration, such as 23.3% in 5 cells per μL. However, by adding the step of microscopic identification (Fig. 4G and Fig. S6†), the mSCP could achieve 100% single-cell transfer with one cell into one container (20 experiments), where only the confirmed single cells were transferred. Finally in order to test the ability of single-cell isolation from a small number of cells, 10^6 MDA-MB-231 cells were suspended in 5 μL cell media. And 12 cells were successfully isolated with an average isolation speed of one cell per minute. During this process, each cell was identified under the microscope. The uncaptured cells were flowed into the \(-P\) port of the mSCP and could be retrieved by disassembling the mSCP-Tip and ADP-Tips for a second round of single-cell isolation.

Conclusions

In conclusion, the mSCP has been demonstrated and validated with single-cell RNA-seq. Compared with the original SCP, the mSCP makes three big improvements, including combination with microscopic identification to achieve 100% efficiency in single-cell isolation, the capability of operating relatively low cell concentrations, and stable and convenient pressure control by a common pipette. The mSCP has been successfully applied to isolate live single cells into nanoliter volumes for RNA-seq. With the feature of convenience, rapidness, and high efficiency, the mSCP has potential to
contribute to widely accessible single-cell biology studies, including clonal generation, PCR analysis, and RNA-seq.

Acknowledgements

We are grateful for the funding support from NIH-R01 DA035868, R01 CA180083, R56 AG049714, and R21 CA191179.

Notes and references

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