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Single-Cell Isolation by Modular Single-Cell Pipette for RNA-Sequencing

Kai Zhang^{a,b}, Min Gao^c, Zechen Chong^d, Ying Li^{a,b}, Xin Han^{a,b}, Rui Chen^c, and Lidong Qin^{a,b,e}

^aDepartment of Nanomedicine, Houston Methodist Research Institute, Houston, TX 77030, USA

^bDepartment of Cell and Developmental Biology, Weill Medical College of Cornell University, New York, NY 10065, USA

^cHuman Genome Sequencing Center, Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030, USA

^dDepartment of Bioinformatics and Computational Biology, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA

^eDepartment of Molecular and Cellular Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA

Abstract

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, SCP-Tip, 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/ μ L 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, which is a powerful tool to isolate single cells for transcriptome analysis.

TOC image

We report a modular Single-Cell Pipette (mSCP), consisting of Air-Displacement Pipette (ADP), ADP-Tips, and SCP-Tip, and its application in single-cell isolation for RNA-sequencing.

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 perturbations¹⁻⁴. The primary step for successful single-cell RNA-seq highly requires a convenient and reliable method to rapidly isolate a live cell into submicroliter suspension and then transfer it into a specific container such as a PCR tube for genetic analysis⁵⁻⁷. However, it is still challenging for micromanipulation⁸⁻¹¹ and fluorescence activated cell sorting (FACS)^{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 diameter⁹) 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 microscopic field. Although relatively accurate, it is time consuming and low throughput⁵. FACS, by contrast, is a fast and automated method for single-cell isolation. However, cell viability and integrity may be affected by high shear force from 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 microfluids¹⁴⁻¹⁶. According to the difference of container for single-cell isolation, these platforms can be divided into three types: 1) 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 surrounding by closing valves; 2) microdroplet-based platform^{20, 21} such as The Chromium Single Cell 3' Solution (10 $\times$ Genomics) where both single cells and barcoded beads are simultaneously encapsulated into rapidly flowing nanoliter-sized aqueous droplets; and 3) microwell-based platform^{22, 23} such as ICCELL8 Single-Cell System (WaferGen) where single cells are randomly trapped into round microscale wells and subsequently confirmed by microscope. These platforms integrate 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 laboratories be 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)²⁴, 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 working pressure empirically and is limited by a relatively high cell concentration (10^3 cells/ μ L). Here, we report a modular SCP (mSCP) which overcomes the above limitations. (1) Pressures are provided by 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 equipping with SCP-Tip containing a hydrodynamic trap, the mSCP enables to isolate single cells from a relatively low concentration of cell suspension. With the new capability, we achieved single-cell isolation by mSCP from 5–10 cells/ μ L cell suspension.

Materials and methods

Design and fabrication of SCP-Tips

The SCP-Tips, including SCP-Tip containing a hook and 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 18- μ m 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 puncher (Harris Uni-Core). Then, the upper PDMS layer (4–5 mm thickness) with microstructure was irreversibly bonded to the bottom PDMS layer (0.5–1 mm thickness) without microstructure by using 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. The NK-92 cells (ATCC) were cultured in Alpha minimum essential medium (ATCC, VA, USA) supplemented with 0.2 mM inositol (Sigma), 0.1 mM 2-mercaptoethanol (Sigma), 0.02 mM folic acid (Sigma), 100–200 U/ml recombinant interleukin-2 (PeproTech, NJ, USA), 12.5% horse serum, 12.5% 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 by cell-free media or PBS and finally adjusted into known cell concentration.

Detailed assembly of mSCP

The detailed assembly of mSCP is as following. (1) Insert an ADP-Tip (20–300 µL, #022491547, Eppendorf) filled with 50–100 µL cell-free liquids into the bottom of +P port of 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 –P port of 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 liquid. (5) Disconnect the ADP with +P ADP-Tip and immerse the end of SCP-Tip into a small container filled of liquid to prevent potential clogging of microchannel at SCP-Tip end due to liquid evaporation.

Detailed operation of mSCP for single-cell isolation

The detailed operation of mSCP for single-cell isolation is as following. (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 pipette to prevent cell sedimentation. (3) Push the piston button down of ADP and connect to the –P ADP tip. (4) Completely release piston button to generate a –P (–200 µL for mSCP-Tip containing a hook and –50 µL for mSCP-Tip containing a hydrodynamic trap) and quickly immerse the SCP-Tip end into 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 ×10 objective. (7) Connect the ADP to the +P ADP-Tip and push 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 single-cell droplet into a desired container. (8) Disconnect ADP from +P ADP-Tip while pushing piston button down to avoid liquid goes 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 surface and then rapidly weighed by a precise electronic balance (XS105 dual range analytical balances, Mettler Toledo). The single-cell droplet volume was calculated by assuming the droplet density of 1 mg/µL.

RNA-seq and data analysis

After single-cell isolation by 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) was 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 human reference genome (hg19) using Tophat2²⁵. The quality of 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 cufflinks²⁶ and CummeRbund (<http://compbio.mit.edu/cummeRbund/>) pipelines²⁷. A scaling scatter plot was to show the relationship between all pairs of genes using R “ggplot2” package²⁸.

Acquisition of images and movies

Bright-field and fluorescence images were obtained by EVOS FL imaging system and T3i Canon camera. Movies were filmed by 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 (Figure 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 mSCP is the SCP-Tip mainly containing a Y-bifurcation microchannel for fluid pressure transmission and a microscale hook²⁹ within the straight microchannel for single-cell capture (Figure 1A and Figures S1–S2). The SCP-Tip was designed using AutoCAD and fabricated by photolithography and polydimethylsiloxane (PDMS) molding techniques. On the top surface of SCP-Tip, two 60 degree ports (-P port and +P port with 1.5 mm in diameter and 5 mm in length) were created for the convenient insertion of two commercially available ADP-Tips (Figure 1B, upper left). Specifically, an empty ADP-Tip is inserted into the -P port as -P ADP-Tip and an ADP-Tip filled with 100 μ L cell-free liquids, such as cell culture media or phosphate-buffered saline (PBS), is inserted into the +P port as +P ADP-Tip (Figure 1B, bottom right). The maximum outside diameter of the inserted ADP-Tips (2 mm) is a little larger than the diameter of pressure port (1.5 mm). Therefore, due to excellent elasticity of PDMS, interfaces between 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 (Figure 1C). In detail, only the -P will be applied by connecting the ADP with the -P ADP-Tip (empty) and only the +P will be applied by connecting the ADP with the +P ADP-Tip (containing cell-free liquid). After completion of assembly of mSCP, the gas in the Y-bifurcation microchannel is easily replaced with the cell-free liquid by applying 200 μ L volume of +P. As a result, a small volume of liquid will flow out and form a submicroliter droplet at the end of SCP-Tip.

Single-cell isolation can be achieved in 3 steps (Movie S1). Step 1: single-cell capture and washing. After applying the -P to the -P ADP-Tip by 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 (Figure 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 (Figure 1C, upper). The residual cells at SCP-Tip end are washed out by dipping SCP-Tip end into cell-free liquids while moving up and down several times (Figure 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 was investigated, including -100 μL , -150 μL , and -200 μL (Figure 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 (Figure S3). Results showed that the average liquid velocity within 10 seconds increased linearly with the applied -P, such as 30 mm/s under the -P of 200 μL . Correspondingly, the average cell velocity evaluated through time-lapse video was 22 mm/s, 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 maintained, such as MDA-MB-231 (breast cancer cell) with diameter of 12–20 μm , NIH 3T3 (fibroblast) with diameter of 10–18 μm , and NK-92 (natural killer cell) with diameter of 9–24 μm (Figure S4). Only one cell was capture 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 PDMS SCP-Tip, the captured single cell by the hook can be easily and clearly identified under a common inverted microscope (Figure 2C). Step 3: Single-cell release and transfer. Due to poor cell adhesion on PDMS surface³¹, 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 (Figure 2D).

To fulfill the 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 viability³². The mSCP can meet all the above requirements. (1) One cell in one container. The average single-cell isolation efficiency by mSCP was proportional to cell concentration, such as 93.3% in 10^4 cells/ μL and 86.7% in 10^3 cells/ μL (Figure 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/ 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/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 submicroliter volume with the average value of 500 nL (Figure 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). Therefore, cell morphology was well maintained after transfer (Figure 2D). To further evaluate cell viability, 20 MDA-MB-231 cells were isolated by mSCP into wells of a 96-well plate and 15 of them finally generated clones after 5 days of culture (Figure 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 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 (Figure 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 consistent well with the literature report³³.

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 (Figure 4A and Figure S5). The trap was compatible with various cells, such as NIH 3T3 with diameter of 13–18 μm , K562 (lymphoblast) with diameter of 12–17 μm , and NK-92 with diameter of 11–20 μm (Figure 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 (Figure 4B). As a result, the single cell will be certainly captured by the trap once it is aspirated into the microchannel by applying the –P to the –P ADP-Tip (Movie S2).

The work flow of SCP-Tip containing a trap for single-cell isolation is similar to the operation of SCP-Tip containing a hook (Figure 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 (Figure 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 (Figure 4E). Therefore, the –P of 50 μL provided by the ADP was selected. Under the effect of such low –P, the morphology of captured single cells could still be well maintained (Figure 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/ μL and

76.7% in 10 cells/ μ L (Figure 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/ μ L. However, by adding the step of microscopic identification (Figure 4G and Figure 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, 106 MDA-MB-231 cells were suspended into 5 μ L cell media. And 12 cells were successfully isolated with average isolation speed of one cell one minute. During this process, each cell was identified under the microscope. The uncaptured cells were flowed into the -P port of mSCP and could be retrieved by disassembling mSCP-Tip and ADP-Tips for a second round of single-cell isolation.

Conclusions

In conclusion, the mSCP has been demonstrated and validated with the single-cell RNA-seq. Compared with the original SCP, the mSCP makes three big improvements, including by the combination with microscopic identification to achieve 100% efficiency in single-cell isolation, capability of operating relatively low cell concentration, 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 a potential to contribute to widely accessible single-cell biology study, including clonal generation, PCR analysis, and RNA-seq.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Assembly of modular Single-Cell Pipette (mSCP). (A) The SCP-Tip mainly contains a Y-bifurcation microchannel and a hook. (B) The mSCP consists of ADP, ADP-Tips, and SCP-Tip. The PDMS SCP-Tip contains two 60 degree ports (upper left) which are well compatible with common ADP-Tips (bottom right). The -P port is connected with an empty ADP-Tip and the +P port is connected with an ADP-Tip filled with 100 μL liquids such as cell culture media or PBS. (C) Applying ADP to generate negative pressure (-P) for single-cell capture and positive pressure (+P) for single-cell release, respectively. Insets showing schematic diagrams of cell flow paths.

Figure 2.

Single-cell isolation by mSCP. (A) Sequential images of single-cell capture and washing. (B) The variation of average liquid velocity in different applied negative pressure. (C) Single-cell identification by putting SCP-Tip along with SCP-Tips on a common inverted microscope. (D) Micrographs showing the identification and transfer of a NK-92 cell. (E) Single-cell isolation efficiency under different cell concentrations. (F) Evaluation of single-cell suspension volume. The average value is 0.5 μ L. (G) Clonal generation of MDA-MB-231 cells by using mSCP and serial dilution (SD), respectively. (H) Representative images of proliferation of an isolated cell by mSCP in a 96-well plate for 4 days. Green numbers represent cell numbers. MDA-MB-231/GFP cells are used.

Figure 3. Correlations between single cells and bulk cells in RNA-seq measurement of gene expression with Pearson correlation coefficient (r). (A) Single-cell 1 versus single-cell 2. (B) Single-cell 1 versus bulk cells. (C) Single-cell 2 versus bulk cells. Relative gene expression was estimated by calculating Fragments Per Kilobase of transcript per Million mapped reads (FPKM). MDA-MB-231 cells are used.

Figure 4.

Single-cell isolation by mSCP from relatively low concentration of cell suspension. (A) The SCP-Tip mainly contains a hydrodynamic trap for single-cell capture. (B) Numerical simulation of pressure profile (colors) and velocity distribution (red arrows) around the trap. (C) Schematic diagram of single-cell capture by applying $-P$ and release by applying $+P$. Flow paths are included. (D) The variation of average liquid velocity in different applied negative pressure. (E) Morphological variation of a MDA-MB-231 cell under different negative pressure. (F) Single-cell isolation efficiency under different cell concentrations. (G) Micrographs showing the identification and transfer of a MDA-MB-231 cell.