Lu Yin ORCID iD: 0000-0001-8280-4392

Taehong Kwon ORCID iD: 0000-0002-6635-2557

Miniature auto-perfusion bioreactor system with spiral microfluidic cell retention device

Lu Yin[1*], Wen Yip Au[2], Chia Chen Yu[3], Taehong Kwon[3], Zhangxing Lai [4], Menglin Shang[5], Majid Ebrahimi Warkiani[6], Roger Rosche[7], Chwee Teck Lim[1,5,8,9], Jongyoon Han [1,3,10,11]

[1]Critical Analytics for Manufacturing of Personalized Medicine Interdisciplinary Research Group, Singapore-MIT Alliance in Research and Technology, 1 Create Way, #04-13/14, Singapore 138602;

[2]Astute Water Pte. Ltd., #02-05, 48 Hillview Terrace, Singapore 669269;

[3]Departments of Electrical Engineering and Computer Science, Massachusetts Institute of Technology, 77 Massachusetts Ave, Cambridge, MA 02139, USA

[4]Department of Mechanical Engineering, National University of Singapore, 9 Engineering Drive 1, Block EA, #07-08, Singapore 117575;

[5]Department of Biomedical Engineering, National University of Singapore, 4 Engineering Drive 3, Engineering Block 4, #04-08, Singapore 117583;

[6]School of Biomedical Engineering, University of Technology Sydney, Broadway NSW 2007, Australia;

[7]Whirlcell LLC. 1 Broadway, Cambridge MA 02142, USA

[8]Mechanobology Institute, National University of Singapore, 5A Engineering Drive 1, T-Lab, #10-01, Singapore 117411;

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[9]Institute for Health Innovation and Technology (iHealthtech), National University of Singapore, MD6, 14 Medical Drive, #14-01, Singapore 117599;

[10]Antimicrobial Resistance Interdisciplinary Research Group, Singapore-MIT Alliance in Research and Technology, 1 Create Way, #03-13/14, Singapore 138602;

[11]Department of Biological Engineering, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139, USA.

* Current affiliation of the author: Bioprocessing Technology Institute, Agency for Science, Technology and Research, 20 Biopolis Way, Singapore 138668

Correspondence and request for materials should be addressed to J.H. (jyhan@mit.edu)
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Abstract

Medium perfusion is critical in maintaining high cell concentration in cultures. The conventional membrane filtration method for medium exchange has been challenged by the fouling and clogging of the membrane filters in long-term cultures. In this study, we present a miniature auto-perfusion system that can be operated inside a common-size laboratory incubator. The system is equipped with a spiral microfluidic chip for cell retention to replace conventional membrane filters, which fundamentally overcomes the clogging and fouling problem. We showed that the system supported continuous perfusion culture of Chinese hamster ovary (CHO) cells in suspension up to 14 days without cell retention chip replacement. Compared to daily manual medium change, 25% higher CHO cell concentration can be maintained at an average auto-perfusion rate of 196 mL/day in spinner flask at 70 mL working volume (2.8 VVD). The auto-perfusion system also resulted in better cell quality at high concentrations, in terms of higher viability, more uniform and regular morphology, and fewer aggregates. We also demonstrated the potential application of the system

for culturing mesenchymal stem cells (MSCs) on microcarriers. This miniature auto-perfusion system provides an excellent solution to maintain cell-favourable conditions and high cell concentration in small-scale cultures for research and clinical uses.

Key words: high cell concentration, miniature, auto-perfusion, microfluidic, cell retention

Introduction

Frequent medium perfusion is critical for maintaining high concentration in suspension cultures to resolve fast nutrient consumption and waste production (Zamani et al. 2018). Medium change in a small-scale laboratory and clinical cell culture with tens to hundreds of milliliters culture volume is commonly performed manually. Frequent manual medium change inevitably increases the risk of culture contamination, and results in cell loss and damage when suspending cells undergo centrifugation. Also, the frequency of manual medium change is often constrained by the working hours of the lab personnel. Therefore, automation of medium perfusion is highly desirable for high concentration and healthy cell cultures.

Automated perfusion systems have been mainly applied to middle to large-scale cell cultures (one to a hundred litres), utilizing tangential flow (TFF) or alternating tangential flow (ATF) membrane filtration for medium replacement (Kelly et al. 2014; van Reis et al. 1991). In recent years, several membrane filtration based systems for small-scale perfusion cultures have emerged in the market. Sartorius Ambr® system supports automated perfusion to parallel bioreactors at 100 to 250 mL volume, which has been used for high-throughput screening of culture conditions and product quality. Repligen KrosFlo® TFF filtration module has the flexibility to process 2 mL to 15 L

sample volume, but it is mainly designed for filtration rather than cell culture applications. Despite various improvements made in the membrane filtration technologies (Daniel J.Karst 2016; Pramanik 2014; Tolbert et al. 1981; van Reis et al. 1997), clogging and fouling remain significant limitations to long-term culture with high cell concentration, leading to deteriorated cell viability and loss of biological products (Kelly et al. 2014). Thus, frequent membrane filter replacement and manual cell bleeding are often needed to maintain healthy culture conditions. However, these operations increase the risk of culture contamination. In addition, membrane clogging is prone to occur with small membrane area and pore size (Stressmann and Moresoli 2008), which imposes further difficulties in downscaling the membrane filtration based perfusion systems for small-scale high-concentration cell cultures. As a result, centrifugation and gravitational settling methods have been commonly used to mimic perfusion models in a semi-continuous manner at a culture volume less than 50 mL (Bielser et al. 2019; Gagliardi et al. 2019; Gomez et al. 2017; Sewell et al. 2019).

Various membraneless cell retention methods, such as centrifugation, gravitational cell settling and hydrocyclone separators, could effectively overcome the clogging and fouling problem of conventional membrane filters. However, they suffer from various practical challenges such as cell damage, separation efficiency, and reliability in long-term continuous operations (Castilho 2015). For example, hydrocyclone requires a high perfusate flow rate, limiting its applications in small-scale cultures (Bettinardi et al. 2020; Pinto et al. 2008). Acoustic-based cell aggregation technology has also been widely explored as a highly efficient cell retention method for perfusion bioreactors systems in cell manufacturing and tissue engineering (Li et al. 2014; Trampler et al. 1994). However, its usage is constrained by cell exposure to the acoustic field and power consumption. Herein, we employed a spiral microfluidic

chip as a cell retention device for continuous perfusion culture systems (Kwon et al. 2017; Warkiani et al. 2015). Cells passing through a spiral channel of micron-scale dimensions can be focused to a stream close to one side of the channel due to the interplay of the lift and Dean drag force (Bhagat et al. 2008; Di Carlo 2009; Kuntaegowdanahalli et al. 2009), rendering the other side of the channel free of cells. This cell focusing phenomenon, driven only by hydrodynamic forces, enables the spiral chip to function as a membraneless filter with a design of two branching outlets, where the cell focusing outlet contains the retentate, and the cell-free outlet contains the permeate. Conventional membrane filters must have pore sizes much smaller than a single cell diameter, but the dimensions of the spiral microchannel for cell focusing are 5 to 10 times larger than the cell diameter, thus effectively eliminating the chance of clogging and enhancing the tolerance of aggregates formation in the suspension. Additionally, in contrast to the large membrane area required for efficient perfusion in membrane filters, the surface area of the spiral microchannel is much smaller (< 10 cm^2), thus minimizing fouling.

Based on the spiral microfluidic cell retention technology, we designed and developed a miniature auto-perfusion bioreactor system with a user-friendly control box, which can be entirely placed and operated inside common-size cell culture incubators. The auto-perfusion rate of the system is primarily determined by the geometry of the spiral microfluidic channel but easily adjustable through a roller clamp to meet different application requirements. We demonstrated this auto-perfusion system outperformed conventional manual medium replenishment for a small-scale suspension culture of Chinese hamster ovary (CHO) cells, in terms of higher cell concentration and viability. We also showcased the system's utility in growing human mesenchymal stem cells (MSCs) on spherical microcarriers. This novel system provides an excellent solution

to manufacturing cells and biologics in small-scale perfusion cultures for research and clinical uses.

Materials and Methods

Design and fabrication of spiral microfluidic cell retention chip.

The spiral microfluidic chip was fabricated from a micro-milled aluminium mold (Whits Technologies, Singapore) using standard soft lithography. The mold was designed using SolidWorks software (Dassault Systèmes, France). The spiral microchannel had one inlet and two outlets, namely inner and outer outlets (Figure 1A).

The spiral chip for CHO cell retention possessed six loops with the diameter increasing from 10.6 mm to 26.2 mm (from inlet to outlets). The cross-section of the channel was trapezoidal with 600 μm width, 80 μm/130 μm inner/outer height, respectively. The inner outlet of the chip was extended straight from the end of the spiral channel with the same width, 600 μm. The outer outlet was 30^{o} deviated from the inner outlet with 480 μm width. The inner to outer fluid ratio was approximately 1:1 at an input flow rate of 1.5 mL/min.

The spiral chip for microcarrier retention had 500 μm (height) × 2000 μm (width) rectangular cross-section. The diameter of the spiral loops was from 10 mm to 58 mm. Both outlets were 2000 μm in width.

The chips were cast by curing liquid phase polydimethylsiloxane (PDMS) elastomer (10:1 mixture of the base and curing agent; Sylgard 184, Dow Corning, USA) in the molds at 80 ^{o}C in an oven. After solidification, 1.5 mm holes were punched at inlet and outlets for tubing access, and a 3 mm-thick PDMS base was irreversibly bonded

to the chip with air plasma treatment (COVANCE, Femato Science, Korea). The assembled chips were allowed to cure at 80 ^{o}C overnight in an oven. The completed chip for CHO cell retention was then trimmed into a suitable shape with a plastic mold to fit into a cartridge designed for the auto-perfusion bioreactor control box. The chip for microcarrier retention was used without the cartridge due to its oversize design.

Polystyrene microspheres of 15.45 μm (Bangs Laboratories, USA) and Cytodex 1 microcarriers of 150-250 μm (Sigma-Aldrich, USA) were used to characterize the focusing behavior of the spiral cell retention chips and determine the optimal flow rate for sorting. Real-time cell, microsphere, and microcarrier retention in the spiral microfluidic chip was recorded by an inverted microscope (IX71, Olympus Co., Japan) equipped with a high-speed CCD camera (Phantom v9, Vision Research Inc., USA). The recorded sorting videos were processed for snapshot images using ImageJ software (NIH, USA).

CHO cell culture

Freestyle CHO-S cells (Thermo Fisher Scientific, Singapore) were cultured in 70 mL of Freestyle CHO expression medium (Thermo Fisher Scientific) supplemented with 8 mM L-glutamine, 1% HT supplement (sodium hypoxanthine and thymidine mixture), 0.5% Pen Strep and 0.2% anti-clumping agent (Thermo Fisher Scientific) in a 125 mL Corning ProCulture spinner flask (Sigma Aldrich, USA) in a common CO_2 incubator (HERAcell 150i, Thermo Fisher Scientific) at 37 ^{o}C. The seeding density was 0.25 million cells/mL. Auto-perfusion rate was set to 210 mL/day, equivalent to 3 vessel volumes per day (VVD). The actual average perfusion rate varied slightly from the set rate (2.8 VVD). Manual medium change was performed every 24 h. CHO-S

cells were subjected to 40 μm cell strainer (Becton Dickinson Bioscience, Singapore) before centrifugation at 100 rpm for 10 min. The supernatant was then aspirated, and 70 mL fresh medium with supplements was used to re-suspend the cells (equivalent to 1 VVD). Both the auto-perfusion and manual perfusion cultures were performed for 14 days; the batch culture of CHO-S cells was performed for 7 days without medium change.

Microcarrier-based MSC culture

Commercial human bone marrow-derived MSCs (RoosterBio, USA) were expanded for two passages in a T-175 cell culture flask (Thermo Fisher Scientific) in a common CO_2 incubator (HERAcell 150i, Thermo Fisher Scientific) at 37 ^{o}C with DMEM medium (10567014, Thermo Fishier Scientific) supplemented with 20% FBS, 1% Pen Strep and 2 mM GlutaMAX (Thermo Fisher Scientific). Passage 3 MSCs (2.5 million) were then suspended in 50 mL of complete medium (with supplements described above) in a 125 mL spinner flask (Corning ProCulture, Thermo Fisher Scientific), together with 50 mg Cytodex 1 microcarrier (Sigma Aldrich) rehydrated according to manufacturer's guidelines. To facilitate the attachment of MSCs to the microcarriers, an intermittent spinning at 25 rpm for 2 min at every half an hour was performed overnight, using a programmable magnetic stirrer (SI-0303 MAGSTIR GENIE, Scientific Industries, USA). The expansion of MSCs on microcarriers was performed at spinning speed of 60 rpm for 7 days. Auto-perfusion rate of MSCs culture was set to approximately 1 VVD (50 mL/day), and started on the third day for another 5 days. To accommodate the larger spiral chip for microcarrier retention, the front and back plate of the bioreactor cartridge was removed, and the chip was horizontally placed outside of the control box. A manual medium change of 50 mL

was performed every other day (equivalent to 0.5 VVD) by stopping spinning, allowing the microcarriers to precipitate for 3 min in the spinning flask, and gently aspirating the supernatant. The MSC-laden microcarriers were sampled at different time points and imaged using confocal microscope (Fluoview FV1200, Olympus, JP) after Hoechst staining (Hoechst 33 253 solution, Thermo Fisher, Scientific, USA).

Cell counting

Approximately 50 µL of CHO cell was sampled every day with a syringe (Terumo Medical, JP) through a free lure lock connector on the bioreactor vessel or the waste bottle. The concentration and viability were then measured with an automated cell counter (TC 20, BD Medical Technology, USA). The average size of the sampled CHO cells was measured by Moxi Z Mini automated cell counter with type M cassette (Orflo Technologies Inc., USA).

MSC-laden microcarriers were sampled in the same way, and transferred into a 96-well plate for microscope imaging (CKX41 Olympus, JP). All MSCs were harvested from microcarrier on day 8 (end of culture) by incubating with 0.1% Pronase (Sigma-Aldrich) at 37 ^{o}C for 20 min, after removing medium and washing with PBS for three times. The final yield of MSC was then counted using an automated cell counter (TC 20, BD Medical Technology). The fold of cell expansion was calculated as the ratio of the final cell number harvested from the microcarriers to the initial cell number seeded.

Calculation of cell retention efficiency and perfusion rate

The volume of waste medium was measured every day. The average daily perfusion rate was calculated as $R_p = V_w / T$, where V_w is the volume of the waste medium and

T is the duration (in minutes) between the two measurements in the adjacent two days. The daily cell retention efficiency was then calculated as $R_r = C_b \times 70 \times 100\% / (C_b \times 70 + C_w \times V_w)$, where C_b is the daily cell concentration in the bioreactor, C_w is the daily cell concentration in the waste bottle, V_w is the daily volume of the waste medium in mL, and 70 (mL) is the culture volume. The waste medium was cleared from the waste bottle every day after measurement. The real-time perfusion rates from a commercial syringe pump (PHD 2000, Harvard Apparatus, USA), a peristaltic pump (Masterflex L/S series, Cole-Palmer, USA), and the auto-perfusion system, were measured using an in-line liquid flow meter (SLi 1000, Sensirion, Switzerland).

Statistics

The statistical significance in this study was evaluated by Student's t-test at $p < 0.05$. All analysis was performed with IBM SSPS Statistics software (IBM, USA).

Others

Please refer to the supporting information of this article for the details on the design and manufacturing of the auto-perfusion bioreactor system, CHO cell culture metabolites analysis, and MSC differentiation.

Results and Discussion

Characterization of spiral microfluidic cell retention chip for CHO cell perfusion

We designed a microfluidic chip consisting of a spiral microchannel with one inlet and two outlets for CHO cell retention in perfusion culture systems (Figure 1A). We achieved cell retention by focusing the cells to the inner outlet of the chip based on inertial focusing principles (Bhagat et al. 2008; Di Carlo 2009), and feeding them

back to the cell culture vessel. Meanwhile, the cell-free waste medium was removed from the outer outlet. Thus, the flow rate of outer outlet represented the perfusion rate. Since the average diameter of CHO cells was measured to be 17.67 μm (Supplement Figure S1A), we tested the focusing of polystyrene microspheres of similar size at different flow rates in the spiral microfluidic chip. The optimal flow rate leading to a tight and stable focusing band was 1-2 mL/min (Supplement Figure S1B).

We defined the CHO cell retention efficiency as the percentage of cells sorted into the inner outlet after passing through the chip. To test the cell retention efficiencies at different cell concentrations, we pumped the CHO cells into the chip using a syringe pump (Figure 1B and 1C). At low concentrations of 1.5 and 3 million/mL, all the cells were sorted to the inner outlet at 1.5 mL/min flow rate at the inlet. A small percentage of cells (< 5%) was observed to spill over to the outer outlet as the cell concentration increased to 6 million/mL. The spilling-over increased with the cell concentration, and the cell retention efficiency significantly reduced to 73% and 62% at 12 and 24 million/mL cell concentrations, respectively.

The spilling-over of the cells into the outer outlet at high cell concentration could be overcome by increasing fluidic resistance at the outer outlet to move the streamline boundary between two output flows closer to the outer wall of the channel. Therefore, more cells can be retained through the inner outlet. We applied additional resistance to the outer outlet tubing using a roller clamp to restrain the flow. Observations from high-speed video recording showed that the cells with a tendency to spill over to the outer outlet underwent a sudden turn at the outlet branching position and mostly went into the inner outlet eventually (Figure 1B). The resulted cell retention efficiencies were larger than 99% at both 12 and 24 million/mL cell concentrations (Figure 1C).

With the resistance applied, the splitting flow rates were approximately 1.35 mL/min and 0.15 mL/min at inner and outer outlets, respectively, whereas, without the additional resistance, both the outlets had 0.75 mL/min (Figure 1D). These results indicated that the cell retention chip was capable of retaining 99% of the CHO cells at 24 million/mL concentration with a perfusion rate of 0.15 mL/min. This is the maximum perfusion rate achievable using the current single chip described in Figure 1A.

The cell retention chip may be multiplexed in different ways to enhance efficiency and capacity. On the one hand, a two-stage serial connection of the outer outlet of one chip to the inlet of another chip would salvage the cells spilled over in the first chip through the second one, thus enhancing the cell retention capacity (Kim et al. 2014; Kwon et al. 2018). On the other hand, multiple cell retention chips connected in parallel would increase the perfusion rate proportionally, and are suitable for large volume possessing (Kwon et al. 2017; Kwon et al. 2018).

Development of auto-perfusion bioreactor system

Based on the spiral microfluidic cell retention technique, we developed a small-scale auto-perfusion bioreactor system for suspension cell culture. The core component of the system is a control box, which allows the users to input the flow rates for cell circulation and medium replenishment (Figure 2A). The spiral microfluidic cell retention chip is integrated into a disposable cartridge, which is then inserted into a socket on the control box during operation (Figure 2B).

During the perfusion culture, cells are grown in a spinner flask connected to the cartridge through silicone tubing (Figure 2C and 2D). The internal pump of the control box continuously feed the cell suspension into the spiral chip in the cartridge

at user-defined flow rates. At appropriate flow rates, the chip would sort all the cells to the inner outlet and circulate them back to the spinner flask while removing cell-free medium through the outer outlet to a waste bottle (Figure 2C).

The spinner flask and a magnetic stirrer are placed on top of a weighing scale, which transmits real-time measures to the control box. The change in the weight reflects the change of the medium volume in the spinner flask. In this study, the control box was programmed to allow the medium volume in the spinner flask to fluctuate within a narrow range of ± 10 mL. Once exceeding the limits, the external pump of the control box will start (when the culture volume falls below the lower limit) or stop (when the culture volume raises above the upper limit) feeding fresh medium into the spinner flask (refer to the Supporting Information). Thus, the average rate of medium replenishment was guaranteed to be approximately equivalent to the perfusion rate.

The entire auto-perfusion system can be set up and operated inside a common-size laboratory CO_2 incubator (Figure 2D). The electrical and mechanical stability of the system in the high humidity incubator environment at 37 ^{o}C was firstly verified by continuous perfusion of water over a period of 14 days. Repeated tests showed that the average daily perfusion rate was maintained between 0.11 to 0.15 mL/min (Supplement Figure S2A).

We then tested the cell retention performance of the automated system. At a high perfusion rate of 0.75 mL/min, the CHO cell retention efficiency of the automated system was found to be lower compared to pumping cells into an isolated chip with a syringe pump, and the difference enlarged with increasing cell concentration (Supplement Figure S2B). This was possibly due to the more significant flow fluctuation generated by the peristaltic pump than the syringe pump (Supplement

Figure S2C), leading to periodic spilling-over of cells into the outer outlet. Nevertheless, when additional resistance was applied at the outer outlet using a roller clamp, the cell retention efficiency of the system reached 100% even at high CHO cell concentration (12.6 million/mL) (Supplement Figure S2B). However, the perfusion rate reduced to 0.15 mL/min.

Long-term CHO cell perfusion culture

We verified the performance of the auto-perfusion bioreactor system by culturing CHO cells for 14 days. The concentration and viability of CHO cells in the spinner flask were compared between the auto-perfusion culture and a parallel culture with daily manual medium change (namely manual perfusion), which is a high medium change frequency in common practice. Results showed that both cultures reached the maximum the cell concentration at 9-10 million/mL. However, the auto-perfusion maintained cell concentration at 8.2 million/mL up to the 14th day of culture, which was 25.6% higher than the 6.1 million/mL maintained by manual perfusion (Figure 3A). Both cultures have well-exceeded the recommended cell density for subculture (1-3e6 cell/mL) by the manufacturer of the CHO-S cells and medium used in this study. The manual perfusion culture showed rapid cell growth at an average of 2.16±0.64 fold daily increase and reached maximum cell concentration on Day 5. In contrast, the auto-perfusion culture experienced slower cell growth at an average of 1.43±0.29 fold daily since Day 2 of culture and reached the maximum on Day 9 (Figure 3A). The slower growth phase might imply cell adaptation to the shear stress under continuous circulation in the auto-perfusion system. The cell concentration in the auto-perfusion culture was lower than the manual perfusion from Day 3 to Day 5, but it was significantly higher from Day 7 to Day 14. Consistent with the cell

concentration, the cell viability in the auto-perfusion culture was lower from Day 1 to Day 4, but higher from Day 7 to Day 14 (Figure 3A). In addition, the CHO cells cultured with auto-perfusion also exhibited more uniform and round morphology with less aggregates in suspension, compared to manual perfusion or batch culture without media exchange (Figure 3E).

During the 14-day culture, the auto-perfusion rate was maintained at 0.11-0.18 mL/min with an average of 196 mL/day (Figure 3B), equivalent to 2.8 VVD, which was 2.8 times faster than the manual perfusion culture (1 VVD). To understand the impact of perfusion frequency on maintaining high cell concentration in the culture, we compared the concentrations of metabolites in the auto-perfusion and manual perfusion cultures after they have reached their respective saturated CHO cell densities. Regardless of the higher CHO cell density in the auto-perfusion culture (8.2 *vs.* 6.1 million cells/mL), the glucose concentration was maintained at higher level, and the lactate concentration was maintained at a lower level in the auto-perfusion culture, as compared to the manual perfusion (Figure 4). This was likely due to the higher perfusion rate enabled by the auto-perfusion system thereby, leading to the higher cell concentration and viability (Figure 3). The pH values and concentrations of glutamine, glutamate, ammonium, sodium, and potassium were found in a reasonable range for both saturated cultures. No significant difference was observed between the two medium replenishment modes (Figure 4).

We also analyzed the cell concentration and viability in the waste bottle of the auto-perfusion system. Very few CHO cells (< 0.007 million/mL) were found for the first 5 days of culture with < 50% viability (Figure 3C). After the cell concentration in the spinner flask reached 7 million/mL on Day 6, the cell concentration and viability in

the waste bottle drastically increased, and were maintained at 0.3-0.5 million/mL and 60-80% viability onwards (Figure 3C). The viability of the cells in the waste bottle was much lower, and the cell size was significantly smaller than those cultured in the spinner flask (Supplement Figure S1A). This was in line with the previous finding that unhealthy and nonviable cells exhibit reduction in cell size (Bortner and Cidlowski 2002; Kwon et al. 2018; Yang and Soh 2012). Dying cells and cell debris were likely to be sorted to the outer outlet of the spiral chip due to much smaller cell size, and they were continuously removed from the bioreactor. Continuous removal of unhealthy cells might have also contributed to the higher cell concentration obtained by auto-perfusion. In contrast, the nonviable cells cannot be effectively removed during the centrifugation in manual perfusion. Therefore, the toxic factors from nonviable cells might generate an unfavorable culture environment for the viable cells (Orrenius et al. 2011).

The daily cell retention efficiency of the auto-perfusion system was about 99% in the early stage ($\leq$ 5 days), and it was maintained around 90% in the late stage of the long-term culture (Figure 3D). This result suggested the lower cell concentration in the auto-perfusion culture at the early stage (Figure 3A) was likely due to slow cell proliferation rather than poor cell retention. The cell retention efficiency in the long-term perfusion culture (Figure 3D) was lower than that obtained from the chip performance test (Figure 1C and Supplement Figure S1B), partly due to the existence of more small-size nonviable cells in the long-term cultures. During the 14-day culture, no replacement of the spiral microfluidic cell retention chip was needed, which eliminated the risk of contamination associated with filter replacement. No clogging or fouling was observed at the end of the culture, and a high cell retention efficiency was consistently maintained throughout the culture period (Figure 3D).

Together, these results demonstrated the utility of the auto-perfusion bioreactor system for lab-scale high-density cell culture. Compared to manual medium change, which usually happens at a frequency of one time per day or less for common practice, the automated system not only saves time and labor with the reduction of contamination risk, but also brings advantages of maintaining higher cell concentration and viability through more frequent media change.

Perfusion culture of MSCs grown on microcarriers

Mesenchymal stem cells (MSCs) and their secretome have been widely investigated for therapeutic uses in recent decades (Cosenza et al. 2018; Cunningham et al. 2018; Galipeau and Sensebe 2018; Reza-Zaldivar et al. 2018; Teixeira et al. 2017; Vizoso et al. 2017). Although MSCs naturally adhere to surfaces, culturing MSCs in suspension has been realized utilizing microcarriers (Goh et al. 2013; Rafiq et al. 2016; Tsai and Ma 2016). We also tested the auto-perfusion system for microcarrier culture of MSCs. A larger spiral microfluidic cell retention chip with a rectangular cross-section was designed specifically for the retention of microcarriers (Figure 5A and Supplement Figure S1C). Similarly, the focusing of microcarriers in this chip was tested at different flow rates. The optimal flow rate which efficiently concentrates all the microcarriers to the inner outlet was found to be 3 mL/min, with additional resistance applied to the outer outlet (Figure 5B and Supplement Figure S1D).

The MSCs were firstly allowed to attach on microcarriers with intermittent spinning overnight and cultured for another day to ensure sufficient spreading and adaptation to the suspension culture. The auto-perfusion was then started at 1 VVD. A parallel culture was subjected to manual media change at every other day (0.5 VVD). We confirmed the growth of MSCs on microcarriers by confocal imaging of nucleus

staining at the beginning and the end of both the manual and auto-perfusion cultures (Figure 5C). The cell expansion with auto-perfusion was 27% faster than the manual media change (Figure 5D). The advantage of auto-perfusion might be more significant when a higher concentration of MSCs are cultured on more microcarriers, because the auto-perfusion system can prevent fast depletion of nutrients. The cultured MSCs were characterized by conventional tri-lineage differentiation assays. Histology staining of Alizarin Red S, Oil Red O and Safranin O demonstrated comparable multi-lineage potencies between the MSCs cultured with auto-perfusion and manual medium change (Figure 5E).

Nevertheless, it should be pointed out that MSC-laden microcarriers might experience high mechanical stresses when repeatedly passing through the spiral chip and peristaltic pump of the auto-perfusion system (Born et al. 1992; Kretzmer and Schugerl 1991). We observed significant cell detachment from the microcarriers and cell death in the case of less MSC seeding density and shorter adaptation time to the suspension culture. It is critical to inoculate higher cell density and allow sufficient cell spreading and extracellular matrix protein production on the microcarriers to ensure firm attachment and resist high shear during circulation. However, the requirements on high initial MSC density would largely limit the fold of expansion per passage of culture. Optimized microcarrier culture conditions for shear-sensitive primary cells in this auto-perfusion system would require further investigations. For example, using partial occlusion peristaltic pumps with convex rollers or centrifugal pumps to reduce shear force to the cells grown on the microcarriers might facilitate the performance of the auto-perfusion system.

Conclusion

In this study, we presented an auto-perfusion bioreactor system with a microfluidic cell retention chip for small-scale culture of suspension cells and adherent cells grown on microcarriers. This system allows automated medium replenishment at a much higher frequency than the practically possible manual replenishment rate, which promotes higher cell concentration and viability. Some critical technical features and performance indicators of this novel cell retention technology are compared to the conventional membrane filtration and acoustic cell trapping techniques (Table 1).

The use of microfluidic inertial focusing for cell retention fundamentally solves the clogging and fouling issues happening frequently to the conventional membrane filters in perfusion bioreactors. However, as the microfluidic cell retention principle determines the range of the microchannel dimensions and the flow rate of the perfusate, the maximum culture concentration and the perfusion rate for a single chip are constrained. We showed that 99% cell retention efficiency could be achieved at 24 million/mL by sacrificing 80% of the perfusion rate (Figure 1B-D), yet the cell retention efficiency is still likely to decrease significantly at higher cell densities. In contrast, membrane filtration can achieve close to complete cell retention despite culture concentration and perfusion rate; acoustic cell trapping can also reach 98% cell retention at above 100 million cells/mL (Table 1).

The common clogging and fouling issues of membrane filtration can be effectively resolved by the microfluidic retention and acoustic cell trapping technologies, leading to more consistent and higher recovery of cell products in the bioreactor. The two membraneless cell retention solutions are also capable of continuously removing dead cells during the perfusion process. Thus, they could promote a healthier culture

condition than membrane filtration which retains all the live and dead cells in the bioreactor (Table 1).

Membrane filtration has well demonstrated its scale-up capability for large-volume cultures, but scale-down of membrane filtration is difficult due to clogging and fouling. Acoustic cell trapping has been mainly demonstrated for small culture volumes (<250 mL). Scaling up to culture volume larger than 100 L and scaling down to less than 10 mL are both challenging with this technique (Table 1). Due to the microfluidic nature of our new cell retention technology, it can be applied to very small-volume cultures, but scaling-up a single chip (i.e., enlarging channel dimensions such as microchannel width and height) for large-volume culture is not possible. Nevertheless, the microfluidic cell retention technology is still applicable to large-volume perfusion by adopting scale-out strategies. Many microfluidic chips can be manufactured in a multiplexed manner to perform perfusion simultaneously, which increases the perfusion rate proportionally. This scale-out approach is much more compatible with the microfluidic technology, as compared to the membrane filtration and acoustic cell trapping, thanks to the micron-level dimension of the spiral microchannel and the cheap manufacturing cost of a single perfusion module (Table 1). In spite of that, the engineering challenges in manufacturing stacked microchannels and balancing flows in multiple channels should not be overlooked.

The presented novel bioreactor system prototype has already integrated the user-interface, cell retention device, and pumps into a miniature control box, but the external magnetic stirrer, culture vessels, and media bottles still occupy considerable space inside an incubator. The long tubing also holds dead volume of the cell suspension, which is difficult to harvest at the end of the culture. Further

improvements in the design and integration of different parts will make it a smaller and more robust product.

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Authorship

L.Y., M.E.W. and J.H. designed research; L.Y., W.Y.A., C.C.Y., Z.L. performed research; T.K., R.R., C.T.L. and J.H. contributed materials, reagents and analytic tools; L.Y. and J.H. analysed data; and L.Y., T.K., M.L.S., C.T.L. and J.H. wrote the paper.

Conflict of interests

Roger Rosche is the CEO of the Whirlcell LLC, which is commercializing the technology. Majid Ebrahimi Warkiani and Jongyoon Han filed patent applications related to the technology presented here, which are being licensed by Whirlcell LLC.

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Tables

Cell retention technology	**Microfluidic**	**Membrane**	**Acoustic**
Driven force	Hydrodynamic	Size-exclusion	Acoustic wave
Maximum cell concentration	20-30 million/mL	200 million/mL [§]	125 million/mL [¶]
Cell retention efficiency	> 90%	~ 100%	98% [¶]
Perfusion rate	Constrained	Flexible [§]	Flexible [¶]
Chance of clogging and fouling	None	High	None
Cell product recovery issue	No	Yes	No
Dead cell removal	Yes	No	Yes
Scale-up capacity (for culture volume > 100 L)	Low [#]	High	Low
Scale-down capacity (for culture volume < 10 mL)	High	Low	Low
Scale-out capacity	High [#]	Low	Low
Cost of one cell retention module (for <250 mL culture volume)	< $ 5	$ 250	$ 7,000

Table 1: Comparison of technical features and performances of different perfusion technologies. [§] Information acquired from Sartorius technical brochures. [¶] Information acquired from SonoSep and Applikon technical reports. [#] Scaling-out the microfluidic cell retention technology by multiplexing a large number of micron-scale spiral microchannels allows for high-throughput perfusion in large-scale cultures.

Figure Legends

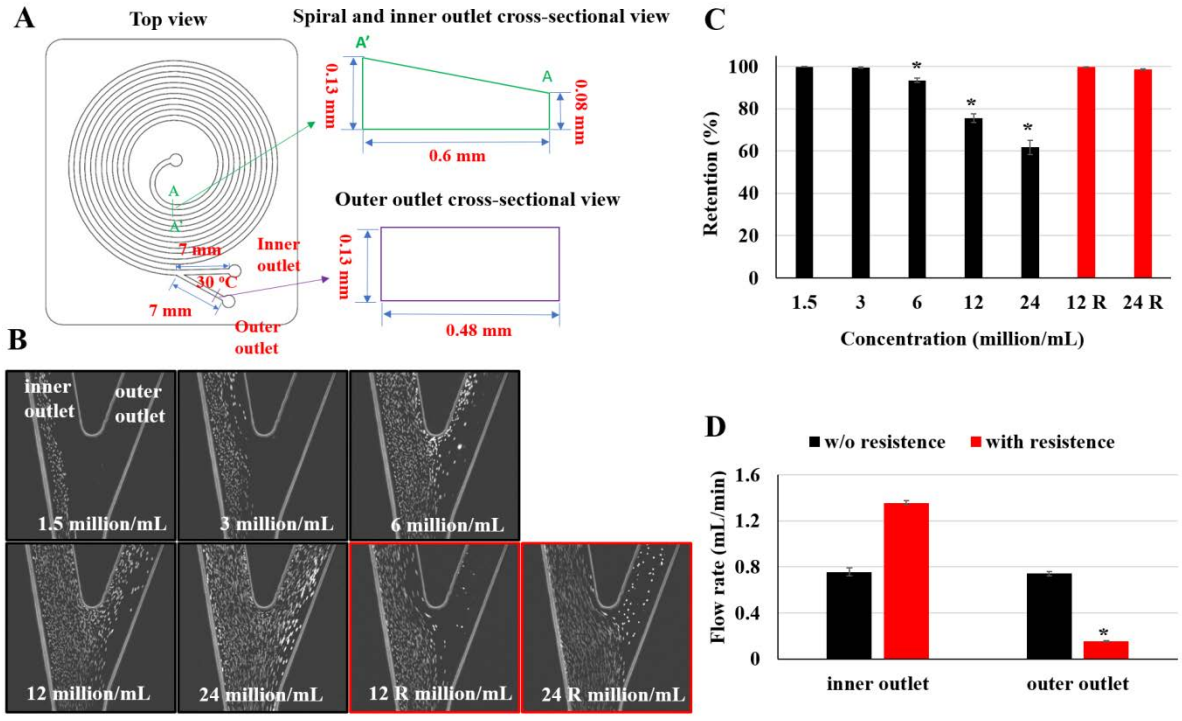

Figure 1: Design and characterization of spiral microfluidic cell retention chip for CHO cell retention. (A) Schematic of spiral microfluidic for CHO cell retention. The spiral channel and inner outlet of the chip had trapezoidal cross-section, and the outer outlet had rectangular cross-section. (B) CHO cell focusing in the spiral microfluidic chip at different cell concentrations captured by high-speed camera. "R" indicates additional fluidic resistance applied at outer outlet of the chip. (C) CHO cell retention efficiency with the chip at different concentrations. * indicates significant

difference in cell retention compared to low concentration at 1.5 million cells/mL. (D) Splitting flow rates (at 1.5 mL/min sorting flow rate) with and without outer outlet resistance. * indicates significant difference in the flow rates from the two outlets. Error bars represent standard deviation.

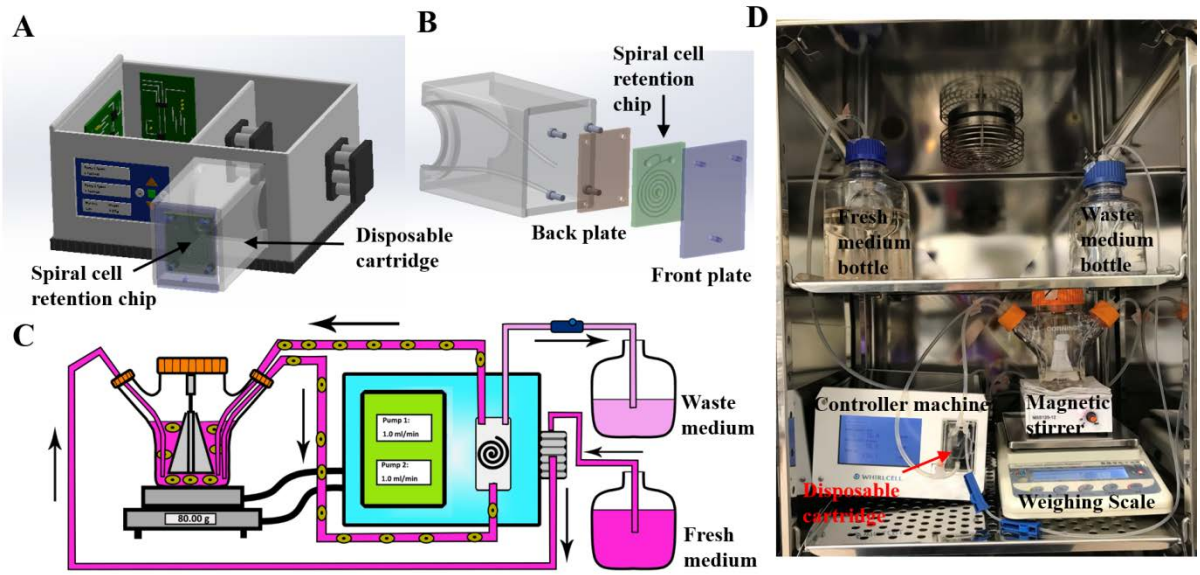

Figure 2: Design, setup and working principle of auto-perfusion bioreactor system. (A-B) Schematics of the controller box (A) and the disposable cartridge with cell retention chip (B). (C) Schematics of the auto-perfusion bioreactor setup and working principle. (D) A picture of the auto-perfusion bioreactor system operating inside a CO_2 incubator.

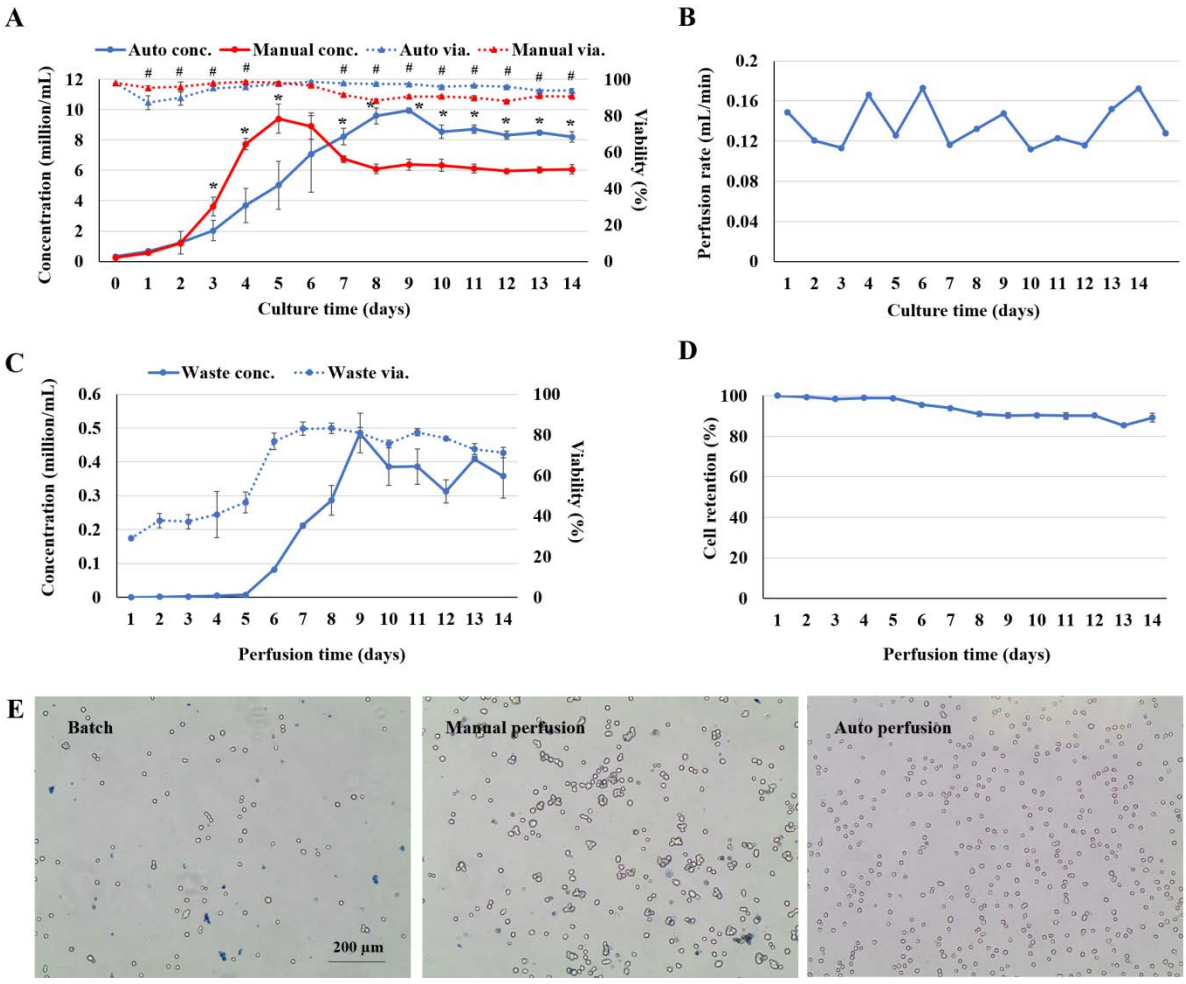

Figure 3: Long-term CHO cell culture using the auto-perfusion bioreactor system. (A) Daily concentration and viability of CHO cells in continuous auto-perfusion culture and manual perfusion culture. (B) Daily perfusion rate of the automated system during continuous culture expansion. (C) Daily concentration and viability of CHO cells in the waste bottle. (D) Daily cell retention efficiency of the automated system during continuous culture expansion. (E) Bright field images of CHO cells stained with Trypan Blue at the end of batch (Day 7), manual perfusion (Day 14) and auto-perfusion culture (Day 14). All error bars represent standard deviation. * indicates significant difference in cell concentration, and # indicates significant difference in viability between auto-perfusion and manual perfusion at different culture time points ($p < 0.05$). Error bars represent standard deviation.

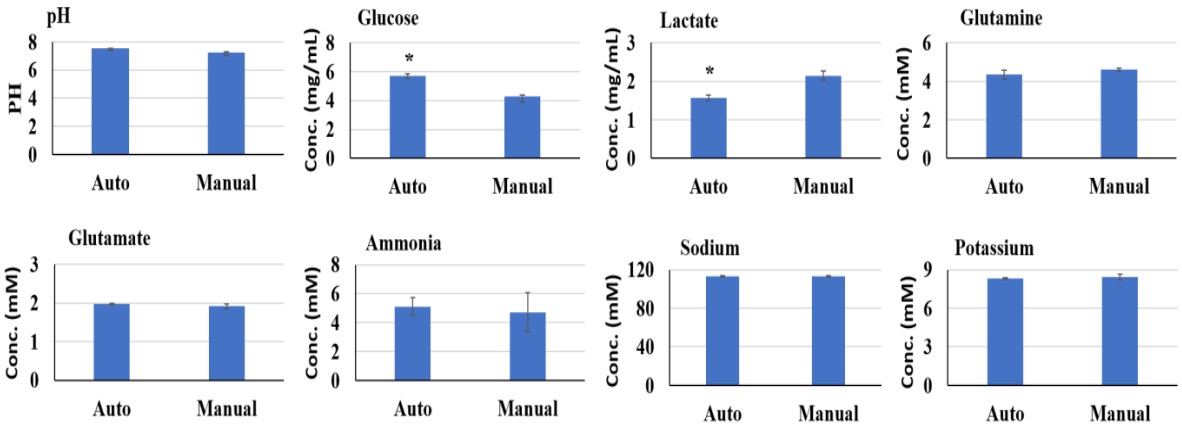

Figure 4: Analysis of pH and metabolites of the auto-perfusion bioreactor system. The pH values and metabolites concentrations of auto-perfusion and manual perfusion culture at respective saturated CHO cell concentrations. * indicates significant difference between auto-perfusion and manual perfusion ($p < 0.05$). Error bars represent standard deviation.

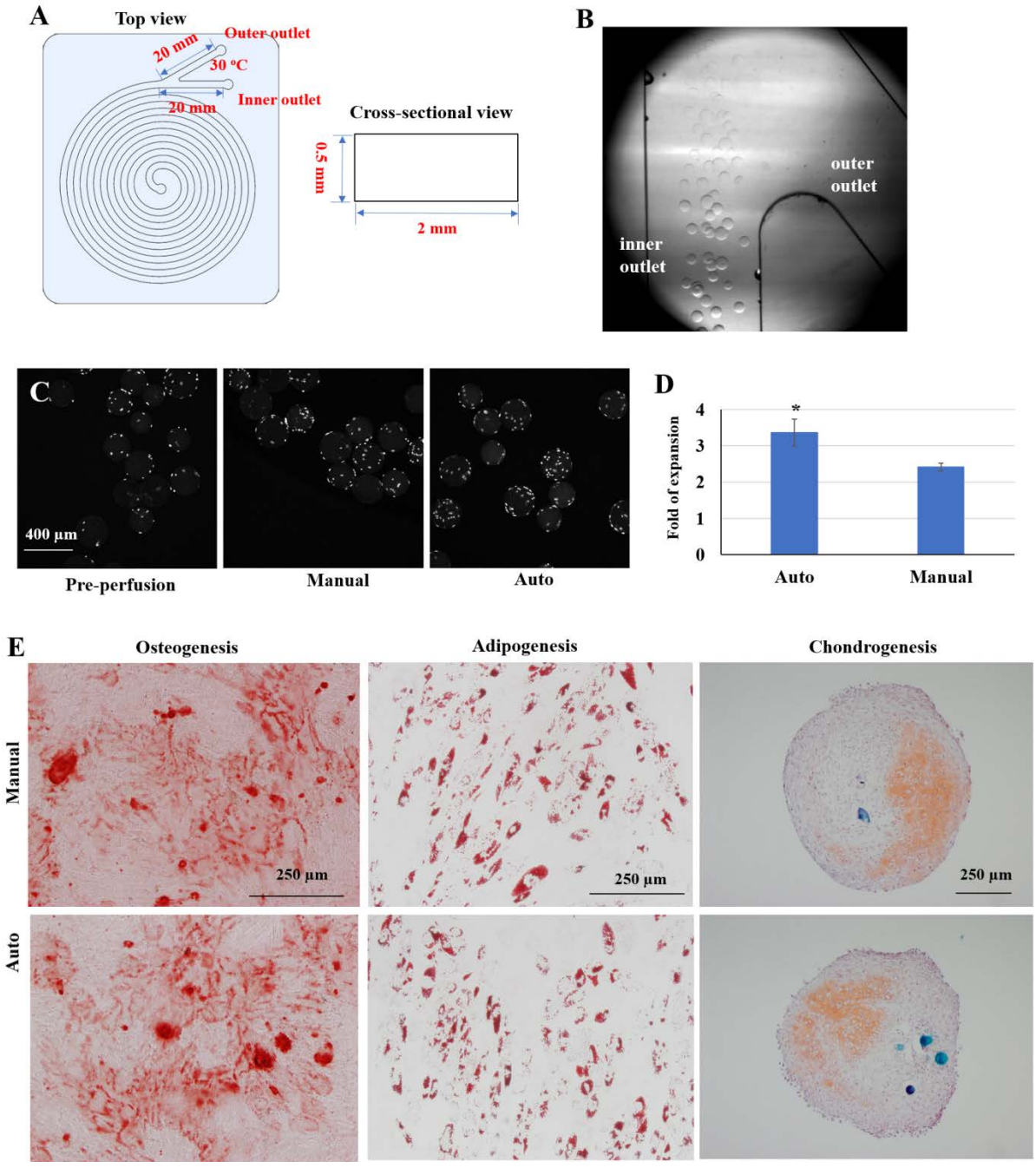

Figure 5: Microcarrier based MSC culture using the auto-perfusion bioreactor system. (A) Schematic of the spiral microfluidic cell retention chip with rectangular cross-section. (B) Microcarrier focusing in the spiral microfluidic captured by high-speed camera. (C) Hoechst staining of the nucleus of MSCs grown on microcarriers before perfusion and at the end of culture with auto-perfusion or manual medium change. Error bar: 400 µm. (D) Comparison of MSC expansion between auto-

perfusion and manual medium change. Error bars represent standard deviation. * indicates significant difference between auto-perfusion and manual medium change ($p < 0.05$) (E) Histological staining of tri-lineage differentiated MSCs cultured with auto-perfusion and manual medium change. Alizarin Red S staining was performed after osteogenesis. Oil Red O staining was performed after adipogenesis. Safranin O staining was performed after chondrogenesis. Error bars: 250 μm.