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Establishing single-cell based co-cultures in a deterministic manner with a microfluidic chip

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Editor, ***Journal of Visualized Experiments***

Dear Editor Nandita Singh,

We have revised the manuscript based on the reviewers' comments of our manuscript (JoVE60202, *A microfluidic platform for multiple single-cell interaction analysis*).

Please feel free to let me know if there is still any questions about the submission.

Sincerely,

Chia-Hsien Hsu, PhD
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TITLE:

Establishing Single-Cell Based Co-Cultures in a Deterministic Manner with a Microfluidic Chip

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KEYWORDS:

Single cell, microfluidics, cell-cell interaction, lab-on-a-chip

SUMMARY:

This report describes a microfluidic chip-based method to set up a single cell culture experiment in which high-efficiency pairing and microscopic analysis of multiple single cells can be achieved.

ABSTRACT:

Cell co-culture assays have been widely used for studying cell-cell interactions between different cell types to better understand the biology of diseases including cancer. However, it is challenging to clarify the complex mechanism of intercellular interactions in highly heterogeneous cell populations using conventional co-culture systems because the heterogeneity of the cell subpopulation is obscured by the average values; the conventional co-culture systems can only be used to describe the population signal, but are incapable of tracking individual cells behavior. Furthermore, conventional single-cell experimental methods have low efficiency in cell manipulation because of the Poisson distribution. Microfabricated devices are an emerging technology for single-cell studies because they can accurately manipulate single cells at high-throughput and can reduce sample and reagent consumption. Here, we describe the concept and application of a microfluidic chip for multiple single-cell co-cultures. The chip can efficiently capture multiple types of single cells in a culture chamber (~46%) and has a sufficient culture space useful to study the cells' behavior (e.g., migration, proliferation, etc.) under cell-cell interaction at the single-cell level. Lymphatic endothelial cells and oral squamous cell carcinoma were used to perform a single-cell co-culture experiment on the microfluidic platform for live multiple single-cell interaction studies.

INTRODUCTION:

Efficient capture of different types of single cells and providing sufficient culture space are needed for single cell co-culture experiments of multiple types of single cells[1]. Limiting dilution is the most commonly used method to prepare the single cells for such experiments, due to the low cost of equipment required. However, due to the Poisson distribution limitation, the maximum single cell acquisition probability is only 37%, making the experimental operation laborious and time-consuming[2]. In contrast, using fluorescence activated cell sorting (FACS) can overcome the Poisson distribution limitation to high-efficiently prepare single cells[3]. However, FACS may not be accessible to some laboratories due to expensive instrumentation and maintenance cost. Microfabricated devices have been recently developed for single cell trapping[4], single cell pairing[5], and single cell culture applications. These devices are advantageous based on their ability to accurately manipulate single cells[6], perform high-throughput experiments, or reduce sample and reagent consumption. However, performing single-cell co-culture experiments with multiple cell types with the current microfluidic devices is still challenging due the limitation of Poisson distribution[1,7,8], or inability of the devices to capture more than two types of single cells[4-6,9,10].

For example, Yoon et al. reported a microfluidic device for cell-cell interaction study[11]. This device uses the probabilistic method to pair cells in one chamber. However, it can only achieve the pairing of two different cell types due to geometric restrictions in the device structure. Another report from Lee et al. demonstrated a deterministic method to capture and pair single cells[12]. This device is increases pairing efficiency by the deterministic method but it is limited by the prolonged operation time required to pair cells. Specifically, the second cell capture can only be performed after the first captured cell is attached to the surface after 24 h. Zhao et al. reported a droplet-based microfluidic device to capture two types of a single cell[13]. We can found that the droplet-based microfluidic device is still limited to the Poisson distribution and can only be used on non-attached cells, and it is not possible to change the culture solution during the cultivation process.

Previously, we have developed a microfluidic "hydrodynamic shuttling chip" that utilizes deterministic hydrodynamic forces to capture multiple types of single-cell into the culture chamber and can subsequently perform cell co-culture experiment to analyze individual cell migration behavior under cell-cell interactions[14]. The hydrodynamic shuttling chip comprises an arrayed sets of units that each contains a serpentine by-pass channel, a capture-site, and a culture chamber. By using the difference in flow resistance between the serpentine by-pass channel and the culture chamber, and a specially designed operation procedure, different types of single cells can be repeatedly captured into the culture chamber. Notably, the ample space of the culture chamber can not only prevent the cell from being flushed during cell capture out but also provide sufficient space for the cells to spread, proliferate and migrate, allowing for observing of live single-cell interactions. In this article, we focus on the production of this device and detailed protocol steps.

PROTOCOL:

1. Fabrication of a wafer mold by soft lithography

NOTE: Mask pattern data is available in our previous publication[14].

1.1. Dehydrate a 4-inch silicon wafer in a 120 °C oven for 15 min.

1.2. Spin coat 4 g of SU-8 2 negative photoresist onto a 4-inch silicon wafer at 1,000 rpm for 30 s to create a 5 µm thick layer (layer #1).

1.3. Soft bake layer #1 on a 65 °C hotplate for 1 min and then transfer layer #1 to a 95 °C hotplate for 3 min.

1.4. Cool layer #1 to room temperature, place it onto the holder of the semi-automatic mask aligner, and align with the layer #1 chrome-plated photomask (capture-site layer).

1.5. Expose layer #1 with 365 nm UV light at a dose of 150 mJ/cm^2.

1.6. Remove layer #1 from the aligner and post-bake on a 65 °C hotplate for 1 min. Transfer layer #1 to a 95 °C hotplate for 1 min.

1.7. Cool layer #1 to room temperature. Immerse in a propylene glycol monomethyl ether acetate solution to wash away the uncrosslinked photoresist for 2 min. Gently dry with nitrogen gas to reveal a layer #1 alignment mark.

1.8. Cover the layer #1 alignment mark by an adhesive tape, spin coat 4 g of SU-8 10 negative photoresist onto the layer #1 at 1,230 rpm for 30 s to create a 25 µm thick layer #2.

1.9. Remove the tape, soft bake layer #2 on a 65 °C hotplate for 3 min, and then transfer layer #2 to a 95 °C hotplate for 7 min.

1.10. Cool layer #2 to room temperature, place layer #2 onto the holder of the semi-automatic mask aligner, and align the layer #2 chrome-plated photomask (bypass channel layer) to the layer #1 alignment mark.

1.11. Expose layer #2 with 365 nm UV light at a dose of 200 mJ/cm^2.

1.12. Remove layer #2 from the aligner and post-bake on a 65 °C hotplate for 1 min and transfer layer #2 to a 95 °C hotplate for 3 min.

1.13. Cool layer #2 to room temperature, and cover the layer #1 alignment mark by adhesive tape. Spin coat 4 g of SU-8 2050 negative photoresist onto layer #2 at 1,630 rpm for 30 s to create a 100 µm thick layer #3.

1.14. Remove the tape, soft bake layer #3 on a 65 °C hotplate for 5 min, and then transfer layer #3 to a 95 °C hotplate for 20 min.

1.15. Cool layer #3 to room temperature, place layer #3 onto the holder of the semi-automatic mask aligner, and align the layer #3 chrome-plated photomask (culture chamber layer) to the layer #1 alignment mark.

1.16. Expose layer #3 with 365 nm UV light at a dose of 240 mJ/cm^2.

1.17. Remove layer #3 from the aligner and post-bake on a 65 °C hotplate for 5 min. Transfer layer #3 to a 95 °C hotplate for 10 min.

1.18. Cool layer #3 to room temperature. Immerse in a propylene glycol monomethyl ether acetate solution to washed away the uncrosslinked photoresist for 10 min, and gently dry with nitrogen gas.

2. PDMS device preparation for multiple single cell capture

2.1. Place the wafer mold and the weighing dish containing 100 µL of trichlorosilane in a desiccator (only for silanization) and apply a vacuum (-85 kPa) for 15 min.

NOTE: Silanize the wafer surface with trichlorosilane to create hydrophobic surface properties before PDMS casting so that it can effortlessly be peeled off from the wafer PDMS mold.

2.2. Stop the vacuum, and then silanize the wafer mold in the desiccator (only for silanization) at 37 °C for at least 1 h.

2.3. Mix PDMS base and PDMS curing agent in a ratio of 10:1. Pour a total of 20 g of mixed PDMS onto the wafer mold in a 15 cm dish.

2.4. Place the 15 cm dish into a desiccator and apply vacuum (-85 kPa) for 1.5 min. Then remove the 15 cm dish from the desiccator. Keep for 20 min at room temperature. Finally, remove residual air bubbles in PDMS with nitrogen gas.

2.5. Place the 15 cm dish in an oven at 65 °C for 2-4 h to cure PDMS.

2.6. Remove the PDMS replica from the wafer mold, and then punch a 1.5 mm inlet and a 0.5 mm outlet on the PDMS using a 1.5 mm inner diameter and a 0.5 mm inner diameter puncher (**Figure 1C**).

2.7. Clean the PDMS replica and the slide surface with removable tape and then treat the surface with oxygen plasma (100 W for 14 s).

2.8. Manually align the PDMS replicas with the slide and bring them into contact with each other.

2.9. Place the PDMS slide in a 65 °C oven for 1 day.

NOTE: Permanent bonding between the slide and the PDMS replica is achieved to form the device.

2.10. Immerse the PDMS device in a container filled with phosphate buffered saline and place into a desiccator. Then apply vacuum (-85 kPa) for 15 min to remove air bubbles.

2.11. Place the PDMS device in a cell culture hood and sterilize the device with UV light (light wavelength: 254 nm) for 30 min.

2.12. Replace the PDMS device buffer with medium (DMEM-F12 basal medium containing 1% antibiotic and 10% fetal bovine serum) and incubate the PDMS device at 4 °C for 1 day. This prevents cells from adhering to the PDMS surface.

3. Preparation of a single-cell suspension

NOTE: Cell types include human lymphatic endothelial cells (LECs), human OSCC TW2.6 cells expressing WNT5B-specific shRNA (WNT5B sh4) and vector control (pLKO-GFP) which were obtained from our previous study[15]. Please refer to our previous publication for detailed cultivation steps.

3.1. Remove the culture medium when the cells achieve 70-80% confluence. Then gently wash the cells with 5 mL of sterile PBS three times.

3.2. Add 1 mL of DMEM-F12 medium containing 1 µM fluorescent dye into WNT5B sh4 and pLKO-GFP cells (use MV2 medium for LECs) and then incubate the cells for 30 min at room temperature.

NOTE: LECs were stained with green chloromethylfluorescein diacetate (CMFDA) Dye, WNT5B sh4 cells were stained with blue 7-amino-4-chloromethylcoumarin (CMAC) Dye and pLKO-GFP cells were stained with red DiI fluorescent dye.

3.3. Gently wash the cells with 5 mL of sterile PBS three times.

3.4. Remove the PBS and add 2 mL of 0.25% Trypsin-EDTA (0.05% Trypsin-EDTA for LECs).

3.5. Incubate the cells for 4 min at room temperature and then gently tap the tissue culture dish to promote cells detachment.

3.6. Add 4 mL of DMEM-F12 medium to disperse WNT5B sh4 and pLKO-GFP cells (For LECs use 3 mL of MV2 medium and 1 mL of trypsin neutralizer solution). Then transfer the cells into a 15 mL tube, and centrifuge at 300 x *g* for 3 min.

3.7. Remove the supernatant, and resuspend the cell pellet in 1 mL of DMEM-F12 medium gently. Count the number of live cells in a hemocytometer by using the standard Trypan Blue exclusion method[16]. Prepare 1 mL of cell suspension at 3 x 10^5 cells/mL concentration in DMEM-F12 medium, and then keep cells on ice to prevent cell aggregation.

NOTE: In order to improve single-cell capture efficiency, careful preparation of the single cell suspension with well-dissociated is required.

4. Multiple single-cell capture and triple single-cell culture

4.1. Connect a poly-tetrafluoroethene (PTFE) tube between the outlet of the device and syringe pump. Remove the medium and add 1 µL of cell suspension at a concentration of 3 x 10^5 cells/mL into the inlet of the PDMS device.

4.2. Load the cell suspension into the device by a syringe pump at a flow rate of 0.3 µL/min (**Figure 2A**). Flow direction is from the inlet to the outlet.

NOTE: Load immediately after adding the cell suspension into the inlet to prevent cell sedimentation.

4.3. Add 1 µL of DMEM-F12 medium into the inlet of the PDMS device after step 4.2. Load the DMEM-F12 medium into the device by a syringe pump at a flow rate of 0.3 µL/min (**Figure 2B**). Flow direction was flowing from the inlet to the outlet.

4.4. Load 0.3 µL of DMEM-F12 medium into the device by a syringe pump at a flow rate of 10 µL/min (**Figure 2C**). Flow direction was flowing from the outlet to the inlet.

4.5. Repeat steps 4.1 to 4.4 to load other cell types into the device.

4.6. After completing the cell capture, use a microscope with 4x lens to image each culture chamber.

NOTE: The fluorescence emissions of the cells was used to identify and count the number of individual cells in each culture chamber.

4.7. Remove the PTFE tube and seal the inlet and the outlet with polyolefin tape to create a closed culture system.

4.8. Move the PDMS device to a 10 cm culture dish and add 10 mL of sterile PBS around the PDMS device to avoid evaporation of the medium from the device.

4.9. Transfer the culture dish to an incubator (37 ° C, 5% CO_2 and 95% humidity) for triple single-cell culture.

4.10. Microscopically observe and photograph cell growth every 12 h.

REPRESENTATIVE RESULTS:

The device has a three-layer structure as shown by the cross-section photograph of a cut PDMS device (**Figure 1A**). The first layer contains a capture-site (6.0 µm in width and 4.6 µm in height) that connects the culture chamber and the by-pass channel. The difference in flow resistance between the culture chamber and the by-pass channel causes the cells to flow into the capture position and fill the entrance of the small path. After a cell is captured at the capture position, the flow resistance of the small path is increased, causing the next incoming cell to go toward and through the by-pass channel to the next downstream capture-site. The serpentine design of the by-pass channel (25.0 µm in width and 26.4 µm in height) of the second layer is used to increase its flow resistance. The dimensions of the culture chamber in the third layer (500.3 µm in diameter and 111.3 µm in height) are designed to provide sufficient space for cell culture experiment, and to reduce the flow rate in the chamber to keep the cells from being flushed out of the chamber.

The tools required for the operation procedure (**Figure 2**) are common in general laboratories, including microscope, syringe pump, glass syringe, centrifuge tube, tubing, and pipette. The device is approximately 1/5 of a coverslip with a 2 mm thickness and is suitable for observing under a microscope with 4x to 20x lens. With this method, one type for single cells can be captured in the culture chambers under 7 min, so the total operation time for triple single cells was less than 21 min. The single cell capture efficiencies of the demonstrated three cells were 70.83% ± 15.42% (LECs), 73.96% ± 14.09% (WNT5B sh4) and 78.13% ± 3.13% (pLKO-GFP), respectively. The triple single cell capture efficiency was 47.92% ± 7.86% (**Figure 3B**). The reflux operation step is used to release the cells from the capture sites simultaneously and flow the cells into the culture chambers (**Figure 2C**). During this process, if a cell is not released from its capture-site, the released cell at its downstream capture-site will not enter the culture chamber due to by-pass channel having a lower flow resistance than the culture chamber. This is the major reason why the HSC has a triple single cell capture efficiency of only 47.92 ± 7.86%, which is still significantly greater than the probability of a Poisson distribution (~5%).

The cell culture results showed that multiple single-cell co-cultures of lymphatic endothelial cells and squamous cancer cells can be performed for 24 h, and the cells' proliferation and morphology can be observed under microscope (**Figure 4, Supplementary Videos 1–3**). In the presence of pLKO-GFP cells, WNT5B sh4 cells and LECs showed better proliferative capacity and showed that the morphology approached lamellipodia. These results demonstrate the ability of this device to high-efficiently capture multiple types of single cells in a culture chamber and provide a sufficient culture space useful for studying multiple single cell type interactions.

FIGURE AND TABLE LEGENDS:

Figure 1. Photograph and microscope Images of hydrodynamic shuttling chip. (**A**) Microscope image of the PDMS sectional view. (**B**) A single cell trapping unit of magnified view. (**C**) Appearance of the chip containing 48 units. Scale bar: 100 µm.

Figure 2. Hydrodynamic shuttling chip operation procedure. (**A**) Due to the high hydrodynamic resistance of the by-pass channel, a single cell is trapped in the capture-site. (**B**) After the first cell is trapped and has occluded the capture-site, the following cells flow toward the by-pass channel due to the increased flow resistance. Use medium to wash remaining cells in channel. (**C**) Reflux the cell into culture chamber. Scale bar: 100 µm.

Figure 3. Single cell capture efficiency of LECs, WNT5B sh4 and pLKO-GFP in the hydrodynamic shuttling chip. (**A**) Microscope image of captured triple single cells in culture chamber. (**B**) The green, blue, and red bars represent the capture efficiency of individual cells, respectively, and the yellow bars represent the capture efficiency of triple single-cell. Scale bar: 100 µm.

Figure 4. Paired single cell co-culture and triple single cell co-culture in the hydrodynamic shuttling chip for 24 h. (**A**) Microscope image of triple single cells co-culture for 0, 12 and 24 h. (**B**) Microscope image of pLKO-GFP and WNT5B sh4 cells co-culture for 0, 12 and 24 h. LECs were stained with green CMFDA Dye, WNT5B sh4 cells were stained with blue CMAC Dye and pLKO-GFP cells were stained with red DiI fluorescent dye. Scale bar: 100 µm.

Supplementary Video 1. Cell Loading

Supplementary Video 2. Cell Refluxing

Supplementary Video 3. Second cell type reflux into the culture chamber.

DISCUSSION:

The intercellular interactions of various cells in the tumor microenvironment play an important role in the progression of the tumor[17]. In order to understand the mechanism of cell-cell interactions, co-culture systems are used as a common analytical method. However, multiple cell types and the heterogeneity of the cells themselves have led to experimental complexity and analytical difficulties.

The hydrodynamic shuttling chip allows multiple single-cell loading in the culture chamber by a deterministic method, without being limited by the Poisson distribution limitation in the dilution method and the microwell platform. By providing a high triple single cell capture efficiency (greater than 45%, the Poisson distribution method is 5%) and demonstrating that in the culture chamber, space is sufficient for cell growth and proliferation (**Figure 4**). Due to its ability to efficiently perform multiple single-cell captures and live cell culture observations with simple setup and protocol, we envision these microfluidic devices as useful tools for an extensive range of applications, including cell-cell interactions between multiple cells[18], drug screening[19], and cancer biology[20]. On the other hand, the device structure is moldable, and the structure and size of the capture-site can be changed and applied to other fields such as microorganisms and plant cells. In theory, our method is also adaptable to establish and track microbial co-cultures (e.g., bacteria, yeasts, etc.).

The main limitation of this approach is that the precision level required for the device fabrication is high. This is mainly because the flow resist of the smallest channel can be dramatically changed if its fabricated dimensions are slightly offset. The control of resistances of the microchannels are crucial for the high-efficiency single-cell capture of the device. On the other hand, during cell culture, there is no closure between the chambers. Therefore, paracrine secretion of cells in the chamber may spread into other chambers to affect other cells. Finally, attention must be paid to the cleanliness of the channel and tubing during the preparation of the device. The culture medium and any buffer used in the experiment need to be filtered to prevent particles and debris from blocking the channel.

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DISCLOSURES:

The authors declare that they have no competing financial interests.

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Figure 1

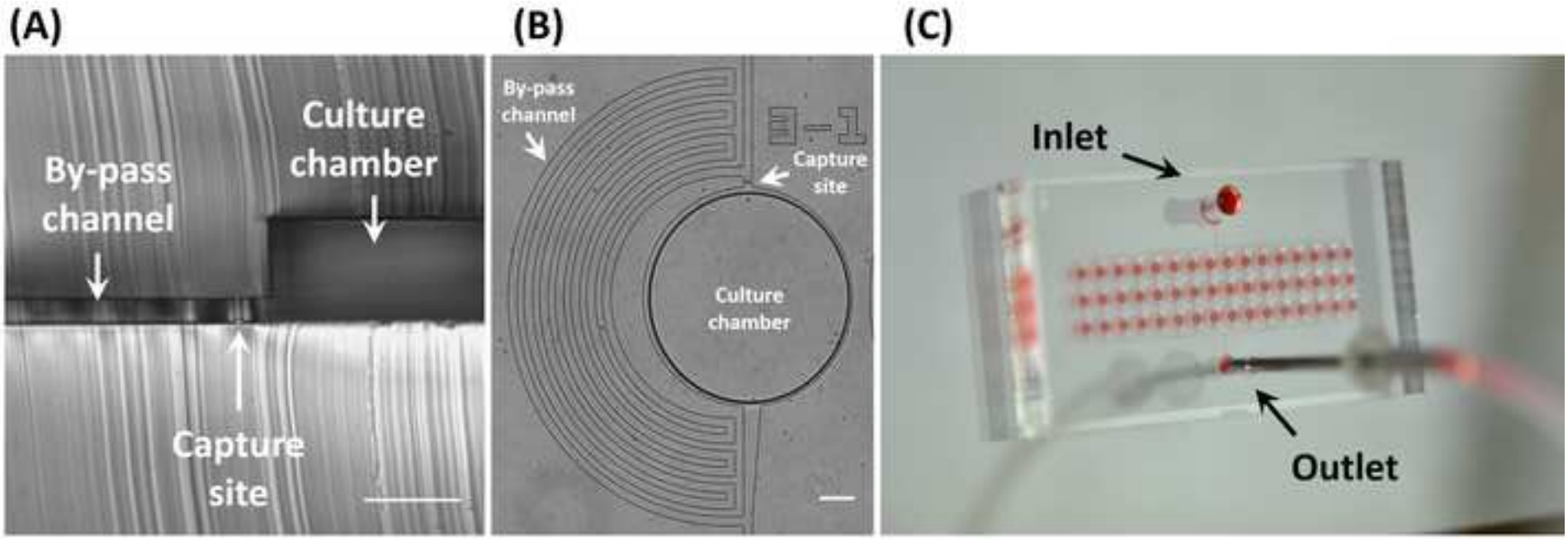


Figure 2

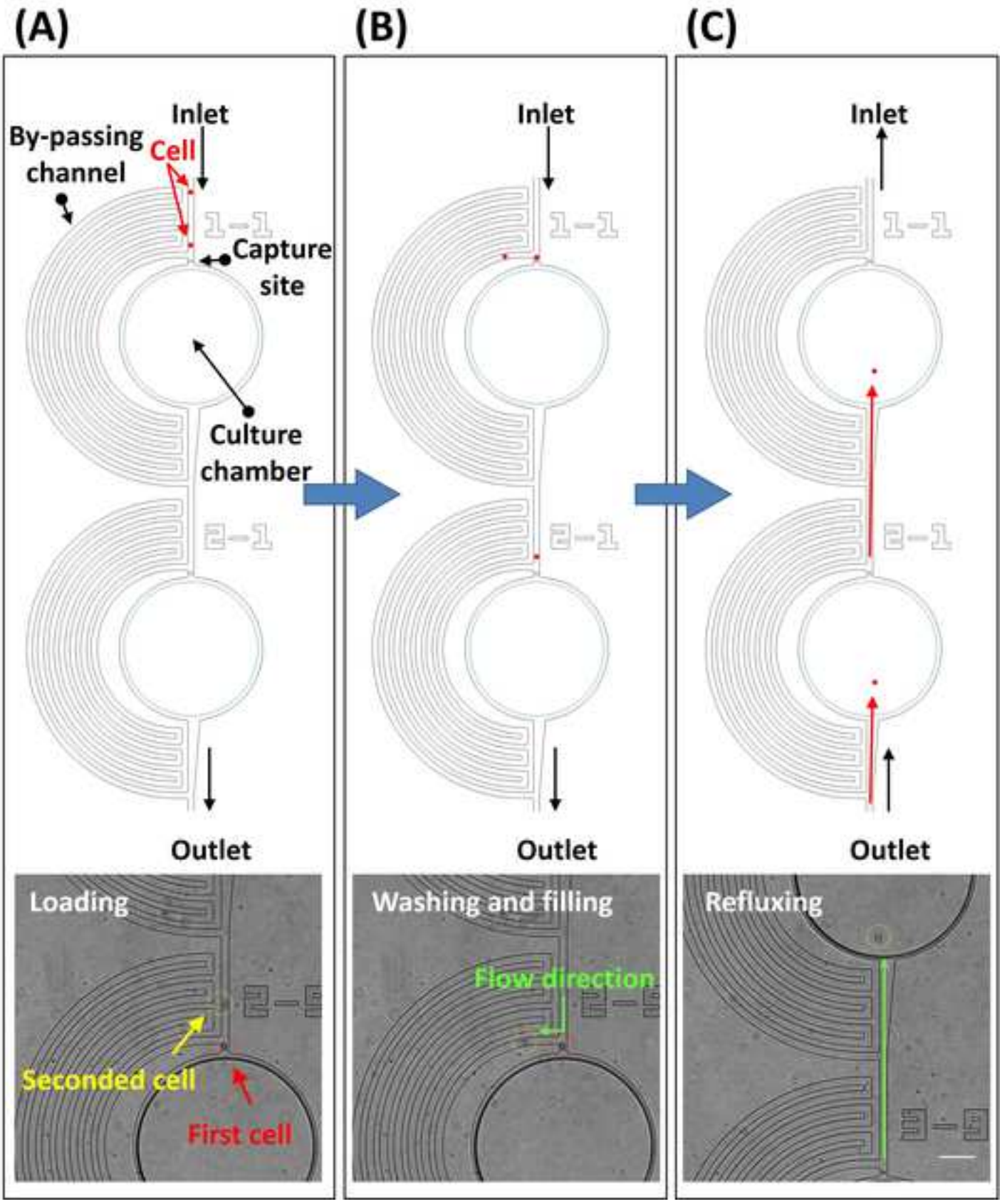
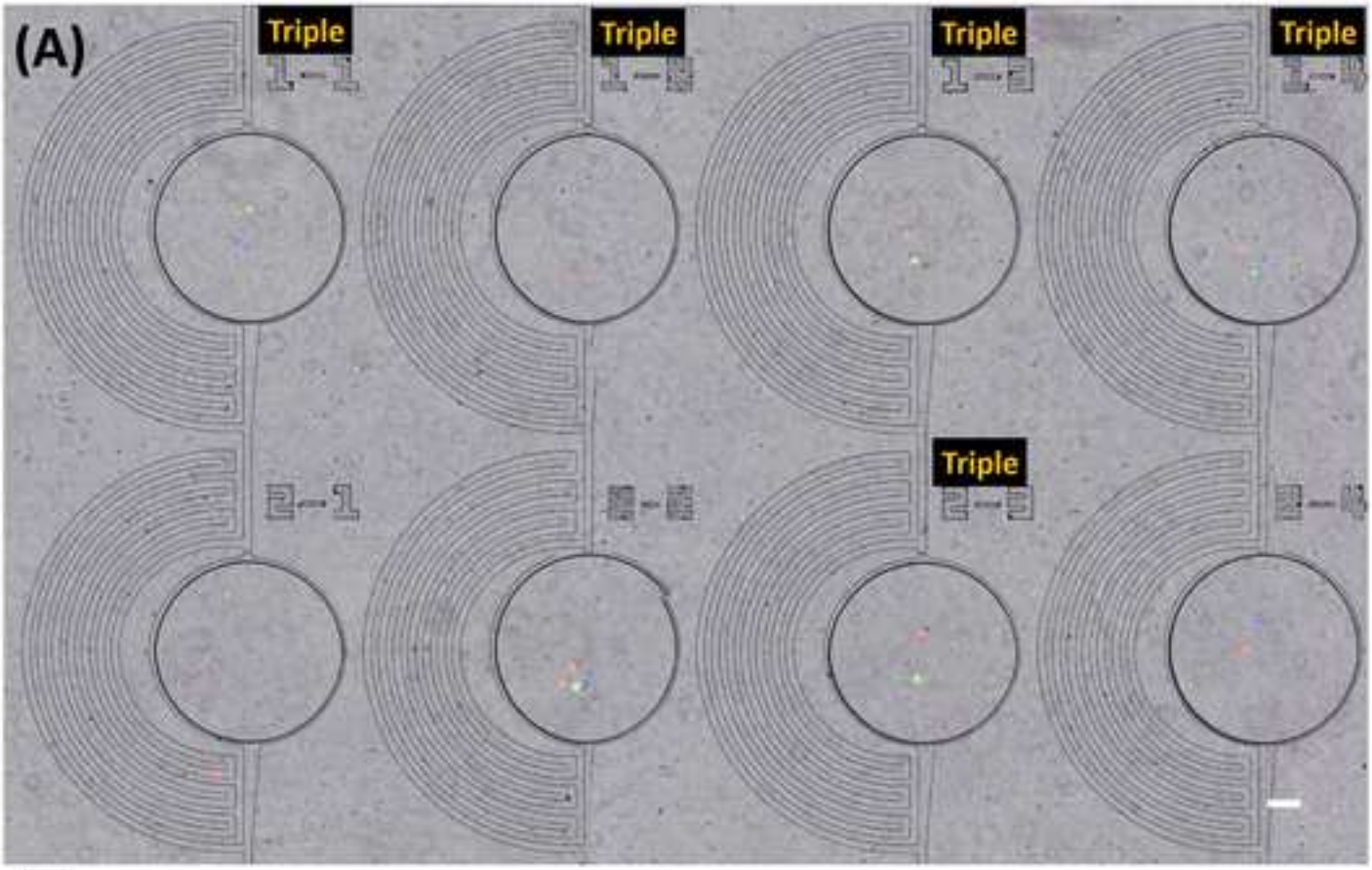


Figure 3



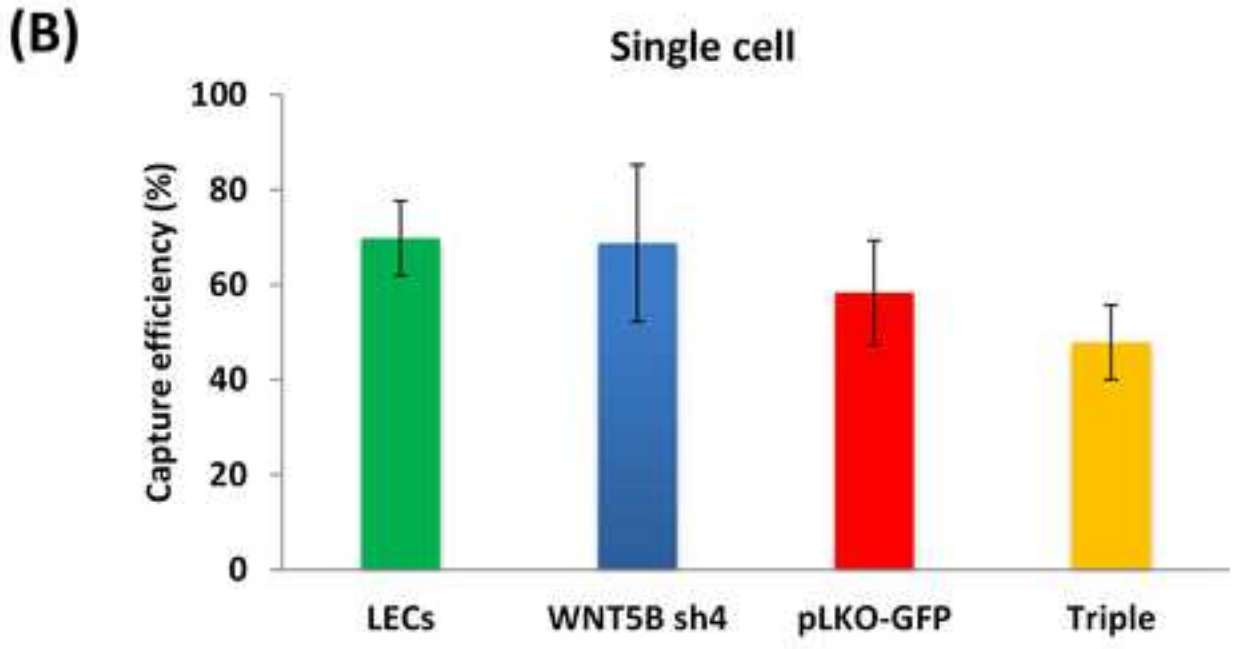
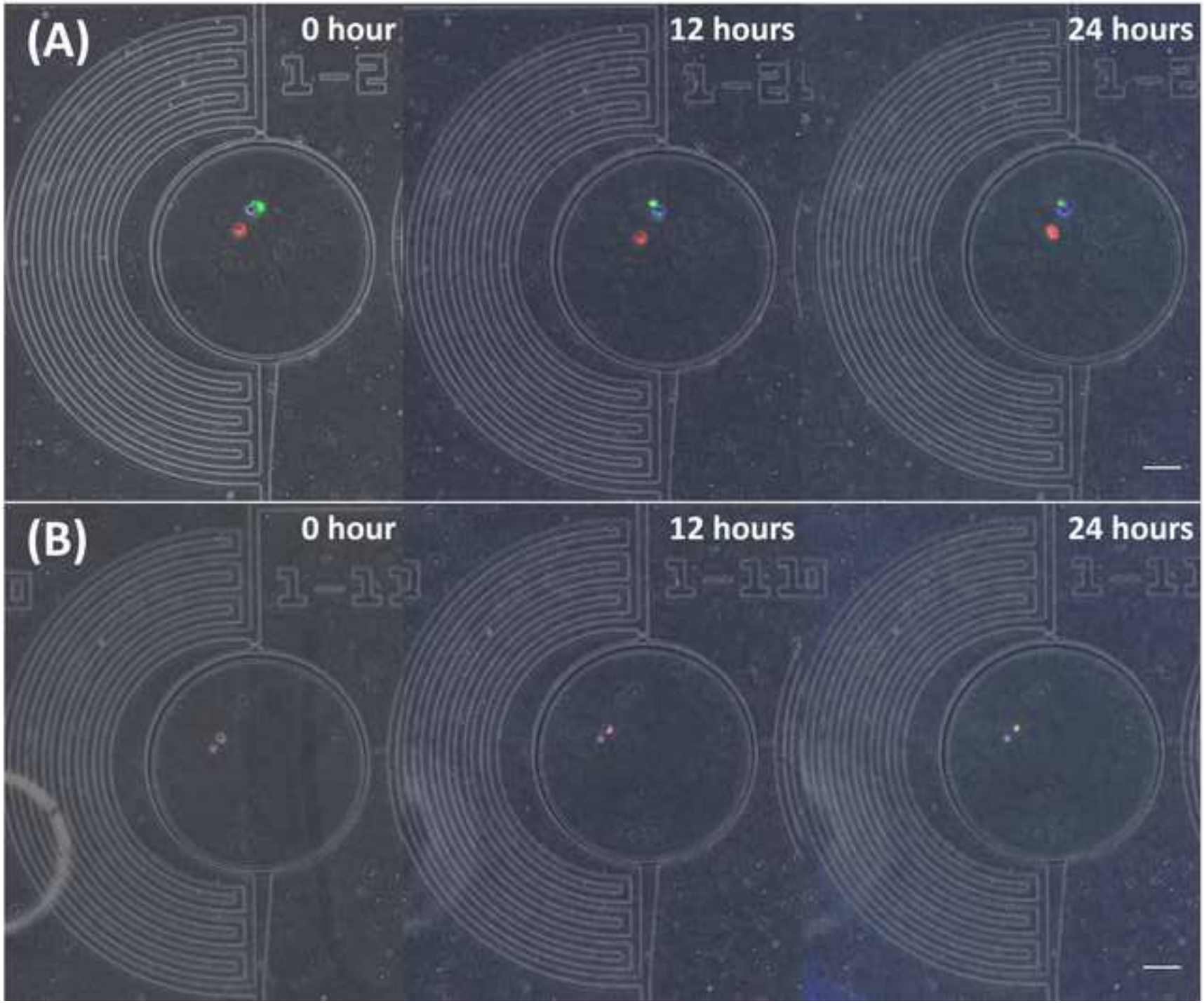
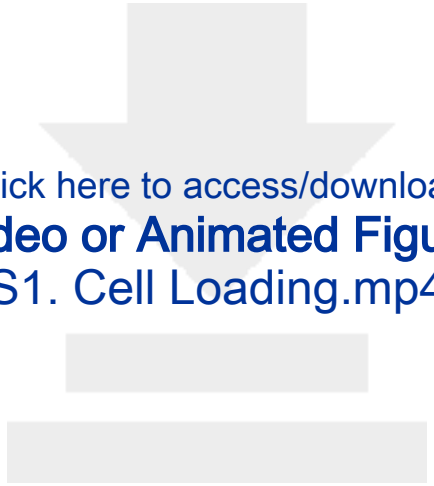


Figure 4



Supplementary Video 1



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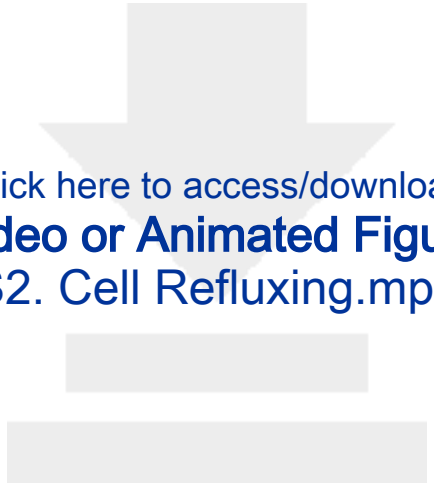
Video or Animated Figure

S1. Cell Loading.mp4

Supplementary Video 2



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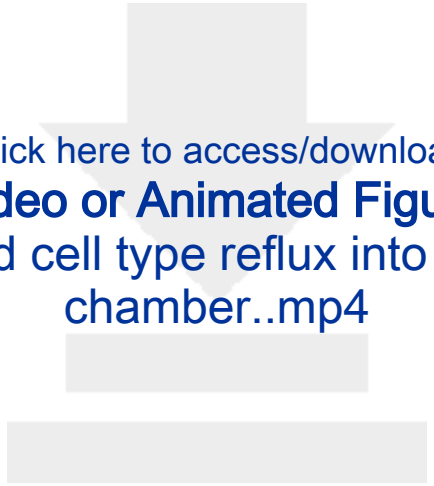
S2. Cell Refluxing.mp4

Supplementary Video 3



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Video or Animated Figure

S3. Second cell type reflux into the culture chamber..mp4

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
3M Advanced Polyolefin Diagnostic Microfluidic Medical Tape	3M Company	9795R	
Antibiotics	Biowest	L0014-100	Glutamine-Penicillin-Streptomycin
AutoCAD software	Autodesk	AutoCAD LT 2011	Part No. 057C1-74A111-1001
CellTracke Blue CMAC Dye	Invitrogen	C2110	
CellTracker Green CMFDA Dye	Invitrogen	C7025	
Conventional oven	YEONG-SHIN company	ovp45	
Desiccator	Bel-Art Products	F42020-0000	Space saver vacuum desiccator 190 mm white base
DiIC12(3) cell membrane dye	BD Biosciences	354218	Used as a cell tracker
DMEM-F12 medium	Gibco	11320-082	
Endothelial Cell Growth Medium MV 2	PromoCell	C-22022	
Fetal bovine serum Hyclone	Thermo	SH30071.03HI	
Hamilton 700 series Glass syringe (0.1 ml)	Hamilton	80630	100 µL, Model 710 RN SYR, Small Removable NDL, 22s ga, 2 in, point style 2
Harris Uni-Core puncher	Ted Pella Inc.	15075	with 1.5mm inner-diameter
Harris Uni-Core puncher	Ted Pella Inc.	15071	with 0.5mm inner-diameter
Hotplate	YOTEC company	YS-300S	
Msak aligner	Deya Optronic CO.	A1K-5-MDA	
Oxygen plasma	NORDSON MARCH	AP-300	

Plasma cleaner	Nordson	AP-300	Bench-Top Plasma Treatment System
Polydimethylsiloxane (PDMS) kit	Dow corning	Sylgard 184	
Poly-tetrafluoroethene (PTFE)	Ever Sharp Technology, Inc.	TFT-23T	inner diameter, 0.51 mm; outer diameter, 0.82 mm
Removable tape	3M Company	Scotch Removable Tape 811	
Silicon wafer	Eltech corperation	SPE0039	
Spin coater	Synrex Co., Ltd.	SC-HMI 2" ~ 6"	
Stereomicroscope	Leica Microsystems	Leica E24	
SU-8 10 negative photoresist	MicroChem	Y131259	
SU-8 2 negative photoresist	MicroChem	Y131240	
SU-8 2050 negative photoresist	MicroChem	Y111072	
SU-8 developer	Grand Chemical Companies	GP5002-000000-72GC	Propylene glycol monomethyl ether acetate
Syringe pump	Harvard Apparatus	703007	
Trichlorosilane	Gelest, Inc	SIT8174.0	Tridecafluoro-1,1,2,2-tetrahydrooctyl. Hazardous. Corrosive to the respiratory tract, reacts violently with water.
Trypsin Neutralizer Solution	Gibco	R-002-100	

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Author(s): Cheng-kun He, Ya-Wen Chen, Ssu-Hang Wang and Chia-Hsien Hsu

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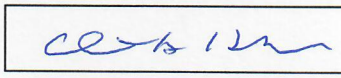
Signature:	
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Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

• **Introduction:** Please expand your Introduction to include the following: The advantages over alternative techniques with applicable references to previous studies; Description of the context of the technique in the wider body of literature; Information that can help readers to determine if the method is appropriate for their application.
Response: We have added descriptions and comparisons of related technologies at line 60 of the revised manuscript.

• **Protocol Language:** Please ensure that ALL text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. “Do this”, “Measure that” etc.) Any text that cannot be written in the imperative tense may be added as a “Note”, however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.
1) Examples NOT in imperative voice: line 77, 79, 81-82 etc.
Response: We have re-modified all the protocol section to allow viewers to understand the steps and add notes to important places. For examples, we have changed the sentence of “The 4-inch silicon wafer was dehydrated within a typical oven at 120 °C for 15 min.” at line 77 of the original manuscript to “Dehydrate 4-inch silicon wafer within an oven at 120 °C for 15 min.” at line 89 of the revised manuscript.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
Response: The protocol already contains various detail and numerical values. However, button clicks and software actions will be different due to different instrument versions. The viewer should refer to the instrument manual and refer to the parameters given by us for the setting.

• **Protocol Numbering:** Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary and all steps should be lined up at the left margin with no indentations. There must also be a one-line space between each protocol step.
Response: We have re-modified the numbering of our protocol. For examples, we have changed the sentence of "**4**.2 The cell suspension was loaded into the channel by a syringe pump at a flow rate of 0.3 µl/min." at line 183 of the original manuscript to "**4**.1.1 Load the cell suspension into the device by a syringe pump at a flow rate of 0.3 µl/min (Figure 2A). (Flow direction was flowing from the inlet to the outlet)" at line 230 of the revised manuscript.

• **Protocol Highlight:** Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps. Please see JoVE's instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.
Response: The experimental steps that will be shown in the video, we have highlight in yellow of the revised manuscript.

1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

Response: We have re-modified the discussion and added future applications at line 342 of the revised manuscript.

• **Figures:** Define all error bars.
Response: We have defined the error bars value in the text. Please see the line 279-281 of the revised manuscript.

• **Figure/Table Legends:** Please add legends for all the videos and ensure that they have been referenced in the text.
Response: We have added legends at line 320-324 of the revised manuscript.

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Comments from Peer-Reviewers:

Reviewer #1:
Manuscript Summary:
In the current manuscript, "A Microfluidic Platform for Multiple Single-cell Interaction Analysis, the authors Hsu et al. have demonstrated the protocol to co-culture multiple single cells on a microfluidic chip to study cellular interactions at single-cell level. To demonstrate the efficacy of their microfluidic chip, they co-cultured oral squamous cell carcinoma and lymphatic endothelial cells and studied the interaction between these two cells. The authors have developed an interesting platform that finds its application for understanding

the communication and interaction between different cell types at single-cell level.

Concerns:
In the manuscript the authors have described the protocol with clarity, however, in certain sections, the description can be clearer. This will help the readers to use the demonstrated protocol for their research in much more effective way. The title and abstract are suitable for the approach that has been demonstrated. The representative results are sufficient and have been discussed well in this manuscript. The authors have also discussed the advantage that this microfluidic platform has over the previously published devices, the drawbacks of the device, and also the potential application. The materials list is also complete. With that I would like to suggest a couple of changes in the manuscript that will make the paper more robust and credible and also help the readers of the journal in continuation of their research work.

1. At certain parts in the manuscript, the authors have talked about Poisson distribution and how this limits the efficiency of co-culturing or pairing multiple cells. However, they do not mention which kind of microfluidic platform takes Poisson distribution into consideration. Is this distribution also valid when other kinds of microfluidic platforms are used for co-culture experiments? The authors can also describe the importance of using microfluidic devices in their introduction section as a number of research groups still prefer to use conventional bulk method based-studies for their research.

Response: We have mentioned the Poisson distribution type of microfluidic devices in the introduction, please refer to reference 1, 7, 8. The importance of microfluidic devices has been mentioned in the introduction, please refer to line 52-55 of the revised manuscript.

2. References: While the authors have made the necessary and appropriate citations, for some examples/lines references can be added. These lines have been listed below:
a. Lines 46
b. Line 52: cell-culture experiment
c. Line 54
d. Line 262

e. Line 264
f. Line 275

Response: Thank you for your suggestion. The necessary references have been mentioned and cited in the article, and the well-known routine method we do not add the reference additionally.

3. In the introduction, the authors have suggested that they have previously developed a HSC chip. In my understanding, the authors are currently, in this manuscript, describing the HSC chip. The authors are required to make changes in the last section of the introduction to make the clarification whether the protocol they are currently demonstrating is a part of the research previously published or this is a new chip design.

Response: The abbreviation of the HSC part has been removed at line 72 of the revised manuscript. We have changed the sentence of “The HSC comprises an arrayed sets of units each contains a serpentine by-pass channel, a capture-site, and a culture chamber.” at line 62 of original manuscript to “The hydrodynamic shuttling chip comprises an arrayed sets of units each contains a serpentine by-pass channel, a capture-site, and a culture chamber.” at line 75 of the revised manuscript. We added the sentence of "In this article, we focus on the production of this device and detailed protocol steps" at line 82 of the revised manuscript.

4. In the protocol section, the authors have with clarity described the steps used for fabrication of the devices. However, to make the protocol section more comprehensible to the readers, the authors should rephrase the steps in the 'Preparation of Single-Cell Suspension' and 'Multiple Single-cell Capture and Triple Single-cell Culture' section. The authors need to explain the steps described in these two sections in more detail or with more clarity.

Response: We have re-modified the 'Preparation of Single-Cell Suspension' and 'Multiple Single-cell Capture and Triple Single-cell Culture' section at line 189, 224 of the revised manuscript to allow viewers to understand the steps and we also added notes to important places.

5. The authors need to add an additional figure that demonstrates the design

of the chip made in AutoCAD. With an AutoCAD drawing (2D and 3D), it will become much easier to visualise the design to the readers.
Response: The design the can be found in our previously published article of this chip, so we do not include it here. .

Reviewer #2:
Manuscript Summary:
This manuscript introduces a microfluidic device that enables co-culture of up to three types of single cells. One cell that reaches in front of a narrow capture channel make the next cells bypass it and go toward the next capture channel. Giving the opposite flow ejects the captured cells from the capture region into larger culture area. Repeating these steps enable loading multiple single cells in culture areas. The protocol in the manuscript covers chip fabrication, cell culture, and handling of the chips.

Major Concerns:
1) The reviewer recommends adding a protocol of visualizing cells in the channels, and the method of validation of the result of cell seeding.

Response: We added Note in step 4.4 at line 244 of the revised manuscript and explained how to shoot and calculate the single cell of the culture chamber.

2) The mask pattern data could be provided as supplementary info to help readers to reproduce the chips and co-culture.

Response: To reproduce the chip the viewers can refer to our previously published article which contains the detailed geometry of the microchannels.

Minor Concerns:
L59
In the context of research that uses cells, some readers could take HSC as a kind of cells. The reviewer thinks HSC is primarily for hematopoietic stem cells. The reviewer suggest not using the acronym for the authors' chip.
Response: We have removed the abbreviation at line 72 and 75 of the revised manuscript.

L72

This note might not be needed as a note to the whole protocol. Instead, it should be moved to material list.

Response: We have removed this note at line 86 of the revised manuscript.

L77

The reviewer thinks a "typical" oven is not a scientific but household one.

Response: Thank you for pointing this out. We have changed the sentence of "The 4-inch silicon wafer was dehydrated within a typical oven at 120 °C for 15 min." at line 77 of the original manuscript to "Dehydrate 4-inch silicon wafer within an oven at 120 °C for 15 min." at line 89 of the revised manuscript.

L78

Does this sentence mean preheating two hotplates to 65C and 95C? Rephrasing recommended.

Response: We consider this step does not need to be specifically stated, so we have removed this step.

L80

The reader may not be familiar with the naming or intent of the layers like "a capture site" at this moment. Therefore, the reviewer recommends that the terms like "a capture site layer" should be summarized beforehand, or just be replaced to names of which the reader does not have to figure out the meaning ("XX um layer" or "Layer #1" etc.).

Response: We have modified the naming to "layer #number" and explained its meaning in the steps. For example, We have changed the sentence of “Place the cooled SU-8 2 coated wafer onto the holder of the semi-automatic mask aligner and align it with the capture-site layer chrome-plated photomask.” at line 83 of original manuscript to “Cool layer #1 to room temperature, place the layer #1 onto the holder of the semi-automatic mask aligner and align with the Layer #1 chrome-plated photomask (capture-site layer).” at line 97 of the revised manuscript.

L82

In this step, May there be another case in which the thickness is not 5um?

Response: We have re-modified the protocol and described the layer thickness in each coating step. For example, We have changed the sentence of “4 g of negative photoresist was coated on a 4-inch silicon wafer by a spin coater; rotated at 1,000 rpm (SU-8 2) for 30 sec to create a capture site layer.” at line 79 of original manuscript to “Spin coat 4g SU-8 2 negative photoresist onto a 4-inch silicon wafer with 30 sec at 1,000 rpm to create a 5 µm thick layer #1.” at line 97 of the revised manuscript.

L84

What part or feature in "it" is aligned with the photomask?

Response: We have re-modified the protocol and described the aligned object in each expose step. For example, We have changed the sentence of “Place the cooled SU-8 2 coated wafer onto the holder of the semi-automatic mask aligner and align it with the capture-site layer chrome-plated photomask.” at line 83 of original manuscript to “Cool layer #1 to room temperature, place the layer #1 onto the holder of the semi-automatic mask aligner and align with the Layer #1 chrome-plated photomask (capture-site layer).” at line 97 of the revised manuscript.

L89

Cooling temperature should be specified.

Response: We have changed the sentence of “After cooling, a 4-inch silicon wafer was immersed in a propylene glycol monomethyl ether acetate (PGMEA) solution, the uncrosslinked photoresist was washed away for 2 min, and then gently dried with nitrogen gas to reveal an alignment mark.” at line 89 of original manuscript to “Cool layer #1 to room temperature, then immersed in a propylene glycol monomethyl ether acetate solution, washed away the uncrosslinked photoresist for 2 min, gently dry with nitrogen gas to reveal a layer #1 alignment mark.” at line 106 of the revised manuscript.

L90

The reviewer thinks the developer of SU-8 photoresist is just PGMEA, not a solution containing PGMEA.

Response: To avoid confusion we have removed the PGMEA abbreviation at line 106 of the revised manuscript.

L114

Step 21 seems to be an operation of the surface profilometer shown in Step 20. The reviewer is not sure if the description in Step 21 is useful. At least Step 21 should be a substep of Step 20.

Response: We considered that this part is not the focus of this publication, so this step has been removed.

L124

Step 2~4 seems to be substeps of Step 1.

Response: We have changed the description of step 1 to note at line 147 of the revised manuscript.

L126,133,147

A desiccator is used in each step. Please clarify all these steps share one desiccator or different desiccators are used for different steps.

Response: We have added instructions in the steps at line 148, 153 of the revised manuscript.

L156

This sentence seems to be somewhat confusing. Please rephrase the sentence so that it clearly shows which cell type is expressing what.

Response: We have re-modified the sentence and add a reference at line 191 of the revised manuscript, the viewers can get more detailed information.

L187,189

The flow direction of the syringe pump flow should be specified.

Response: We have added a description of the flow direction at line 231, 238, 241 of the revised manuscript.

Reviewer #3:

Summary

The protocol "A Microfluidic Platform for Multiple Single-cell Interaction Analysis" by Hsu et al. describes a method for using a microfluidic

Lab-on-a-Chip device for parallel and deterministic co-cultivation of mammalian cells and tracking the interactions of individual cells.
The protocol includes a description for preparing the wafer mold, the PDMS device, the cell suspension and the cell capturing and release process. The authors exemplarily demonstrate their methodology by establishing single cell-based co-cultures of LECs, pLKO-GFP and WNT5B sh4 cells.
Key conclusion
The presented methodology allows studying cellular interactions at the single-cell level based on hydrodynamic principles in order to bring two or more types of different cells together in a confined, microstructured cultivation space. In contrast to other established methods for preparing single cell-based co-cultures, the presented method is deterministic and allows establishing parallel single-cell co-cultures with high efficiency.
Language
The manuscript suffers partially from imprecision in the language. Interestingly, some paragraphs appear well written and comprehensive, while others are difficult to read. I recommend careful copy-editing before publishing.

Response: We have re-edited and corrected the imprecision statement.

Questions
How does the presented methodology compare to other co-cultivation technology, such as deterministic droplet-based co-cultures, in terms of efficiency? Are the cells still alive inside the culture chamber? Is there any harm or alteration to cell physiology expected due to mechanical stress? Is it possible to perfuse the captured cells with different types of media and thereby change the chemical cultivation conditions?

Response: At present, there is no related microfluidic device demonstrate of capturing exceed three types of single cells for co-culture, and the droplet-based co-culture device is not suitable for the experiment of attaching cells (the cells we studied are attached cells). Figure 4 demonstrates that cells are still alive in the culture chamber within 24 hours. The mechanical stress did not show significant cell damage or death under 24-hour culture observation. Our device is capable of replacing different culture fluids to observe cell proliferation and development.

Quality guidelines

Are the title and abstract appropriate for this method article?

*I recommend modifying the title and highlight the possibility to establish single-cell based co-cultures on-chip in a deterministic manner (similar to the title of the original manuscript by He et al. that has been published in Lab-on-a-Chip).

Response: We think this suggestion is a great idea. We have changed the title to "Establishing single-cell based co-cultures in a deterministic manner with a microfluidic chip".

Are there any other potential applications for the method/protocol the authors could discuss?

*Is the presented method also adaptable to establish and track microbial co-cultures (bacteria, yeasts)? Please shortly discuss this aspect.

Response: Thanks for the suggestions. In theory, the capture-site of our device can be modified to fit the size of the microorganism. However, when the size the structures gets smaller, the fabrication becomes more difficult. We have added this to the discussion of the manuscript.

Are all the materials and equipment needed listed in the table? (Please note that any basic lab materials or equipment do not need to be listed, e.g. pipettes.)

*No, please add specifications of chemicals and machines.

Response: We have modified the material list.

Do you think the steps listed in the procedure would lead to the described outcome?

*Yes, but only with some try and error. I see room for improvement (see below).

Response: We have re-modified the protocol sections.

Are the steps listed in the procedure clearly explained?
*No, I recommend to add sub-structures to the individual protocol sections and add a schematic graphical representation of the individual preparation steps

Response: We have re-modified the protocol sections. For example, We have changed the sentence of “The cell suspension was loaded into the channel by a syringe pump at a flow rate of 0.3 µl/min.” at line 183 of original manuscript to “Load the cell suspension into the device by a syringe pump at a flow rate of 0.3 µl/min (Figure 2A). (Flow direction was flowing from the inlet to the outlet)” at line 230 of the revised manuscript.

Are any important steps missing from the procedure?
*No

Are appropriate controls suggested?
*No, please add some quality control heuristics to the protocol, especially for the mold preparation.

Response: We have re-modified the protocol sections.

Are all the critical steps highlighted?
*No, the protocol would benefit a lot from clearly highlighting important or critical steps during the mold and device preparation. Also, the cell loading protocol should be explained in detail and on the basis of a figure.

Response: We have re-modified and highlighting important or critical steps of the protocol sections. The cell loading protocol has been clearly illustrated in Figure 2.

Is there any additional information that would be useful to include?
*Yes, please specify the machines and devices that have been used in detail.

Response: We have modified the material list.

Are the anticipated results reasonable, and if so, are they useful to readers?
*Yes and No. The demonstrated time-lapse imaging of a co-cultivation (Figure 4) does not show any usable result. For what can the method be specifically used? Are the cells growing or interacting? Is it possible to demonstrate the successive filling of the capture sides with a video?

Response: Figure 4 shows that in the presence of LECs cells, the morphology of WNT5B sh4 cells and pLKO-GFP cells tends to be lamellipodia and exhibits better cell growth. This indicates that cell-cell interactions between the three cell types differ from cell-cell interactions between the two cell types. This helps us to clarify the role of different types of cells in co-culture.

Yes, it is possible to demonstrate the successive filling of the capture sites with a videos.

Are any important references missing and are the included references useful?
*No

Conclusion:
At its current state, I cannot recommend accepting the manuscript for publication in JoVE. However, the manuscript should be considered for publication after a major and careful revision. Results that are more meaningful should be added to the manuscript (for example measuring cell interactions, quantifying growth rates of the cells in co-culture and as single cells, etc.). Consulting a professional language service could be of help for improving readability and precision of the protocol.