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Title:

Generation of Dynamical Environmental Conditions using a High-throughput Microfluidic Device

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Keyword:

Microfluidic, high throughput, live cell imaging

Summary:

We present a microfluidic system for high throughput studies on complex life machinery, which consists of 1500 culture units, an array of enhanced peristaltic pumps and an on-site mixing modulus. The microfluidic chip allows for the analysis of the highly complex and dynamic micro-environmental conditions in vivo.

Abstract:

Mimicking in vivo environmental conditions is crucial for in vitro studies on complex life machinery. However, current techniques targeting live cells and organs are either highly expensive, like robotics, or lack nanoliter volume and millisecond time accuracy in liquid manipulation. We herein present the design and fabrication of a microfluidic system, which consists of 1,500 culture units, an array of enhanced peristaltic pumps and an on-site mixing modulus. To demonstrate the capacities of the microfluidic device, neural stem cell (NSC) spheres are maintained in the proposed system. We observed that when the NSC sphere is exposed to CXCL in day 1 and EGF in day 2, the round-shaped conformation is well maintained. Variation in the input order of 6 drugs causes morphological changes to the NSC sphere and the expression level representative marker for NSC stemness (i.e., Hes5 and Dcx). These results indicate that dynamic and complex environmental conditions have great effects on NSC differentiation and self-renewal, and the proposed microfluidic device is a suitable platform for high throughput studies on the complex life machinery.

Introduction:

High throughput techniques are crucial for biomedical and clinical studies. By parallelly conducting millions of chemical, genetic, or live cell and organoid tests, researchers can rapidly identify genes that modulate a bio-molecular pathway, and customize sequential drug input to one's specific needs. Robotics[1] and microfluidic chips in combination with a device control program allow complex experimental procedures to be automated, covering cell/tissue manipulation, liquid handling, imaging, and data processing/control[2,3]. Therefore, hundreds and thousands of experimental conditions can be maintained on a single chip, according to the desired throughput[4,5].

In this protocol, we described the design and fabrication procedure of a microfluidic device, which consists of 1500 culture units, an array of enhanced peristaltic pumps and on-site mixing modulus. The 2-level cell culture chamber prevents unnecessary shear during medium exchange, which ensures an undisturbed culture environment for long-term live cell imaging. The studies demonstrate that the proposed microfluidic device is a suitable platform for high throughput studies on the complex life machinery. Moreover, the advanced features of the microfluidic chip allow automated reconstitution of highly complex and dynamic microenvironmental conditions in vivo, like the everchanging cytokines and ligands compositions[6,7], the completion of which takes months for conventional platforms like 96-well plate.

Protocol:

1. Microfluidic chips design

1.1. Design the microfluidic multiplexer consisting of 18 inlets, each of which is controlled by an individual valve and a peristaltic pump. To increase the liquid volume driven by per pumping cycle, have the peristaltic pump be composed of 3 control channels, which was purposely widened to 200 µm, and 10 connected flow lines.

1.2. Design the shear-free culture chamber. Replication of the 2-level culture unit is composed by a lower cell culture chamber (400 µm x 400 µm x 150 µm) and a higher buffer layer (400 µm x 400 µm x 75 µm), which prevents unwanted shear stress on cells during medium exchange (**Figure 1**).

1.3. Design high-throughput features. Duplicate the culture unit to form a 30 x 50 matrix layout, occupying an area of approximately 7 cm by 5 cm in size.

2. Chip fabrication and operation

2.1. Fabrication of the replica molding using UV lithography

NOTE: The replica molding was fabricated on silicon wafer according to the standard photolithography protocol[8].

2.1.1. Fabrication the channel structures

2.1.1.1. Spinning photoresist: Spin coat 5 mL of the SU-8 3025 negative photoresist on a silicon wafer at 500 rpm for 10 s and 3000 rpm for 30 s.

2.1.1.2. Soft bake: Put the wafer on a hotplate at 65 °C for 2 min and then 95 °C for 10 min, Cool it down to room temperature.

2.1.1.3. Alignment and curing: Fix the wafer and mask on the holder of the aligner and turn on the light source for 18 s to cure the exposed photoresist.

2.1.1.4. Pre post exposure bake: Ramp up the wafer to 95 °C at 110 °C/h from room temperature and keep it at least 40 min till removing.

2.1.1.5. Develop: Dip the wafer in the developing solution (SU-8 developer) and agitate it for 2.5 min to wash off redundant photoresist and get the 25 μm-high channel structure.

2.1.1.6. Hard bake: Cover the wafer with glass Petri dish and bake at 65 °C for 2 min, then ramp up to 160 °C at 120 °C/h and keep it for 3 hours.

2.1.2. Fabrication of the cell culture chamber with the buffer layer

2.1.2.1. Use the parameters of step 2.1.1.1 to spin coat 7 mL of the SU-8 3075 negative photoresist on the above wafer.

2.1.2.2. Soft bake (described in step 2.1.1.2) the wafer and change the mask to align well with markers on the wafer. Then turn on the light source for 24 s to cure the exposed photoresist.

2.1.2.3. Dip the wafer to the developing solution (SU-8 developer) and agitate it for 4 minutes and 40 seconds to wash off redundant photoresist and get a 75 μm-high layer structure that around the 25 μm-high channel structure fabricated before. Hard bake (described in step 2.1.1.6) the wafer to make the complex structure stronger.

2.1.2.4. Repeat steps 2.1.2.1-2.1.2.3 to fabricate a 75 μm-high chamber structure that stack on the layer structure.

2.1.3. Fabrication of the valve structures

2.1.3.1. Using the parameter of step 2.1.1.1 to spin coat 5 mL of the AZ 50x positive photoresist on the above wafer.

2.1.3.2. Soft bake (described in step 2.1.1.2) the wafer and make the mask aligned well with markers on the wafer, then keep the light source on for 20 s and off immediately for 30 s. Repeat the light on/ off procedure in 5 circles to cure the exposed photoresist.

2.1.3.3. Dip the wafer to matched developer and agitate it for about 8 min to wash off redundant photoresist and get the round-shaped valves that overlap with the control channels to ensure good connection. Hard bake (described in step 2.1.1.6) the patterned wafer to make the whole model stronger.

2.2. Microfluidic chip production using soft lithography

2.2.1. Treat the patterned and blank silicon wafers with rimethylchlorosilane for 15 min.

2.2.2. Prepare 3 portions of PDMS gel (10:1 of monomer/catalyst ratio) corresponding to 50 g of flow layer, 20 g of control layer 2, and 20 g of membrane, respectively.

2.2.3. Cast 50 g of PDMS gel on the patterned silicon wafer, and de-gas them for 1-2 hours in the vacuum chamber at -0.85 MPa to copy the flow layer.

2.2.4. Degas the 2 portions of 20 g of PDMS and spin it on the patterned wafer and a blank silicon wafer at 2000-2800 rpm for 30 s as to prepare a control layer and membrane layer.

2.2.5. Put the PDMS-covered wafers into ventilating oven for 60 min at 80 °C for incubation.

2.2.6. Align and bond the different layers together through customized optical device (zoom in 100x) and plasma etching machine. Then keep it in a ventilating oven for 2 hours at 80 °C to enhance the bonding of the chip.

2.2.7. Punch the inlet holes on the chip, and then bond it onto a PDMS-coated coverslip and cured for at least 12 hours at 80 °C before use.

2.3. Chip operation

2.3.1. Connect miniature pneumatic solenoid valves to the control layer of the chip, and open the customized MATLAB graphical user interface[8] to link and control the switch.

2.3.2. Set the closing pressures of push-up PDMS membrane valves to 25 psi.

2.3.3. Deliver dynamically changing combinatorial/sequential inputs to designated chambers (**Figure 2d**) by timely on-off of the valves.

3. Generation of dynamic inputs in cellular microenvironments

3.1. Chip treatment and cell loading

3.1.1. Maintain the standard culture conditions (37 °C, 5% CO_2) on the microscope for at least 5 hours.

3.1.2. Fill the chip with coating medium (i.e., mentioned in the NOTE) and incubate it at the standard culture conditions (described in step 3.1.1) for at least one hour.

3.1.3. Flush the chip by phosphate-buffered saline (PBS) or cell culture medium (Dulbecco's Modified Eagle's medium, DMEM) to build a healthy culture environment.

3.1.4. Harvest cells at 80% confluency, and resuspend the cells using culture media (DMEM) at a density of ~ 10^6/mL. Then load cells into the chip by pressurizing the cell-containing solution.

NOTE: Culturing different cell lines on chip requires corresponding coating medium to treat the cell culture chamber. Typically, for experiments on 3T3 fibroblast and adherent culture of hen all valves controlling the culture chambers are open, cells flow into all culture chambers within the same column.

3.2. Setup for high throughput live-cell imaging

NOTE: For image acquisition, an inverted microscope with an automated translational stage and a digital complementary metal-oxide semiconductor (CMOS) camera were used. The stage and image acquisition were controlled via the customized software.

3.2.1. Visualize the matrix of culture chambers using 10x objective lens in bright field to affirm and define the location coordinates of each chamber of the 30 by 50 chamber matrix.

3.2.2. Transform the objective lens to the 20x or 40x, then select the location coordinates of the desired chamber and the translational stage moves to the assigned position after confirmation. Fine tune the x, y, z focal plane to get an optimal image.

3.2.3. Optimal the light intensity, expose time, and other imaged parameters were determined individually for each channel (i.e., bright-field and fluorescence imaging).

3.2.4. Set the interval and duration of imaging cycle, save the path and then start imaging.

Representative Results:

The conventional on-chip peristaltic pump was firstly described by Stephen Quake in 2000, using which the peristalsis was actuated by the pattern 101, 100, 110, 010, 011, 001 [8,10]. The number 0 and 1 indicate “open” and “close” of the 3 horizontal control lines. Studies using more than 3 valves (e.g., five) have also been reported[11]. Even though the peristaltic pump composed by 3 control lines and 3 flow lines provides nanoliter accuracy, the transportation rate is too slow to feed 1,500 culture chambers. To solve the problem, we include more flow lines (i.e., the 10 connected channels) to increase the liquid volume driven by per pumping cycle (i.e., 101, 100,

110, 010, 011, 001). Thus, the nutrients and drugs can be effectively delivered to designated chambers. The peristaltic pump transported liquid volume can increase by 16x to ~ 50 nanoliters per pumping cycle. As the array of peristaltic pumps is controlled by 3 connected control channels (**Figure 1b**), the solutions from each inlet are delivered simultaneously to the chip and allow instantaneously mixing. Combinatorial and sequential inputs can, therefore, be generated by timely on-off of the inlets connected to different solutions. Using the same methodology, dynamic varying cytokine and ligand concentrations can also be generated. For example, selected combinations of 1, 0.9, 0.2, 0.05, 0.01 g/L and 4 blank culture media can generate a sine wave fluctuation (between 0 to 0.5 g/L) in concentration with step sizes ranging from 0.0005 to 0.01 g/L.

For the culture of primary cells, it is crucial to maintain a stable microenvironment. Shear stress and exhaustion of conditioned medium during medium exchange will affect cellular behavior and cell fate[12]. To overcome these problems, we design a buffer layer to prevent unwanted shear stress on cells during medium exchange (**Figure 1c**). Unlike the conventional culture units, the main advantages of the device (i.e., valve-controlled) are their capabilities of on-site mixing, delivery and maintenance of independent conditions. In **Figure 2d**, we demonstrated that a complex Chinese word can be created on chip via precise delivery of liquids to designated positions and maintenance of an unaffected condition in individual culture chambers. The capabilities of the active fluidic device in on-site mixing are illustrated with a video clip, showing the dynamically changing FITC concentrations in a culture chamber (**Figure 3c** and **Video** 7:47-7:50), which is accomplished by controlling the pumping rate of 2 independent peristaltic pumps connected to 2 inlets[12]. Numerical simulation suggests that when the medium is directed top-down through the culture chambers, shear flow quickly reaches the bottom of the culture well (Video 4:07-4:25). The shear forces can be effectively prevented when the solution is directed from left to right. Even at an input flow rate of 10 mm/s, cell or micrometer-sized tissue remains undisturbed at the bottom of the culture unit.

As is shown in **Figure 3b(1)**, each inlet is control by a valve other than the lower peristaltic pump. When one drug is selected to be delivered into designated chambers, the inlet connected to the drug is open and the operation of the array of the peristaltic pump transports only this drug. For 2 or multiple drugs, we simply open the connected inlets to generate a mixture of drugs at equal volume. We also integrate one independent peristatic pump independent from the array (**Figure 2g**), which could operate at a different pumping rate from the array and therefore generate different dilutions. To mimic the in vivo NSC dynamic environmental conditions, sequential and combinatorial condition of 6 ligands (i.e., *Jagged*, *DLL*, *EGF*, *PACAP*, *CXCL*, *PDGF*), which consists of 720 and 56 different conditions, were generated using the array of peristaltic pumps and delivered to the designated chambers. In detail, sequential conditions can be represented as S_{ij} = {ligand *i* is added on day *j*} and a combinatorial condition as C_i = {ligand *i* is present}, where ligand *i* = *Jagged*, *DLL*, *EGF*, *PACAP*, *CXCL*, *PDGF* and *j*=1,2,3,4,5,6. Responses of the NSC cells and spheres were recorded every 2 hours during culture and stimulation.

NSC spheres are maintained in the 400 μm by 400 μm cell culture chamber (**Figure 2e** and **Figure 4**). Differentiation and stemness (i.e., self-renewal) of the stem cells are represented by the

expression level of *Dcx* and Hes5, respectively. We observed that when the NSC sphere is exposed to CXCL in day 1 and EGF in day 2, the round-shaped conformation is well maintained and there is obvious increase in the sphere size. NSCs with high-*Dcx* expression level die on the 3rd day when EGF is replaced by PACAP, suggesting effects on the differentiated cells[13-16]. Cells start to attach to untreated PDMS surface upon stimulation by DLL on the 4th day, and dissociate into individual cells with the addition of PDGF on the 5th day and Jagged on the 6th day. Changes in the input order of these 6 ligands bring distinctive NSC status (**Figure 5**). For example, the evolvement of NSCs varies dramatically when CXCL switches position with PDGF along the sequence (**Figure 5a and 5b**). These results demonstrate that the dynamic varying environmental conditions have great effects on NSCs differentiation and self-renewal, which paves the way for developing brain-on-chip platforms for biomedical studies as well as clinical applications.

Figure 1: Design of the high-throughput microfluidic chip. a. The enhanced peristaltic pump consists of multiple flow channels and widened control channels, which leads to increase in the transferred liquid volume per pumping cycle. **b.** The array of peristaltic pumps is controlled by 3 connected control lines. Therefore, the inclusion of different inlets at programmed time points can generate combinatorial, sequential and dynamic varying input signals. **c.** To prevent unwanted shear flow, we designed a 2-level culture unit. Cells are maintained at the bottom of the lower level, which is 150 μm in depth. **d.** Each culture chamber is connected to 4 channels, allowing solution to be directed through top-down and left-right directions.

Figure 2: Fabrication of the high-throughput microfluidic chip. a. Microstructures of control and flow layers were firstly patterned on silicon wafer, on which PDMS is casted for replication. **b.** The control, membrane and flow layers are aligned and bonded together using plasma etching and thermal bonding. **c-f.** The advanced features of the chip are demonstrated using green, blue and yellow food dyes. We show that by timely on-off of the valves, solutions of nanoliter accuracy can be delivered to the designated chambers. **g**. The array of peristaltic pumps is controlled by 3 connected control lines (i.e., Control-1), and one independent peristaltic pump controlled by other 3 control lines (i.e., Control-2).

Figure 3: A schematic showing the active fluidic device. A schematic showing that (**a**) cells flow through the bypass channel; (**b**) valves control the inlet and the peristaltic pump; and (**c**) cells flow into the culture chambers.

Figure 4: Representative images of the NSC sphere when being stimulated by sequential drug input. Bright field (top row), Hes5-GFP (second row), *Dcx*-Desred (third row) images show that upon sequential stimulation, substantial cell death occurs among both *Dcx*-high and Hes5-high cells. The emergence of the dark area suggests the death of stem cells, which localize mostly at the core region. Scale bar: 100 μm.

Figure 5: Variations in NSC sphere conformation, *Dcx* and Hes5 expression level, when being stimulated by different sequential inputs. It is demonstrated that variation in the input order of 6 drugs causes changes in tissue, morphological, and expression level of signaling molecules, indicating the sensitivities of NSC sphere to the dynamic environmental conditions. The input

sequences: (**a**) DLL>> PACAP>> EGF>> CXCL>> PDGF>> Jagged, (**b**) DLL>> PACAP >> EGF >> PDGF >> CXCL >> Jagged, (**c**) DLL >> PACAP >> PDGF >> CXCL >> EGF >> Jagged, (**d**) PDGF >> CXCL >> PACAP >> EGF >> DLL >> Jagged. Scale bar: 100 μm.

Discussion:

Various microfluidic devices have been developed to perform multiplexed and complex experiments[17-20]. For example, microwells made of an array of topological recesses can trap individual cells without the use of external force, showing advantageous characters including small sample size, parallelization, lower material cost, faster response, high sensitivity[21-24]. Droplet and surface tension confined droplet eliminate the need for auxiliary hardware such as a micropump, making the transportation of droplets more low-cost and eco-friendlier[25,26]. Drops of liquids can be readily deposited on the surface by micropipettes or sprayer nozzles, and after the experiments, the systems may be facilely refreshed and reusable[27,28]. The slipchip technique allows multiplexed reactions without the involvement of integrated pumps or valves, and therefore user- and producer-friendly[29,30]. However, these systems face a number of technical hurdles, such as storing nanoliter solutions in micro wells, controlling humidity, and limitations of parallel low volume liquid-dispensing technologies.

In this paper, we described a protocol for high throughput biomedical experiments using live cell imaging system and a customized microfluidic device. The procedures of chip fabrication including UV and soft lithography, and setup parameters for automatic fluorescent imaging are demonstrated in detail. The results show that the advanced functional features (i.e., the 2-level culture chamber and enhanced peristaltic pump) of the proposed microfluidic chip outperforms previously reported devices and meet the needs to conduct thousands of parallel experiments. We also described the crucial parameters (e.g., the maintenance of conditioned medium) that one should carefully control to maintain healthy environments for the culture of primary and stem cells.

Cell-based biomedical experiments, which include complex protocols and programmed addition of chemicals, are often deemed time-consuming and laborious. For example, besides the cell manipulation procedures, combinatorial inputs of 6 drugs with hourly refilling requires at least 250 pipetting steps per hour, and repetition till the end of the experiment. Furthermore, modeling dynamic environmental conditions in vivo requires timely addition of one or multiple cytokines, ligands and drugs at the accuracy of seconds, which is impossible for manual operations. We herein demonstrate that by connecting the inlets of the chip to multiple drugs, or different concentrations of single drug, combinatorial, sequential and dynamically varying input signals can be generated and maintained in the shear-free cellular environments. The morphological and chemical responses of cells and tissues, which are maintained in the parallelly running culture chambers, can then be monitored in real-time using the commercial microscope software.

With increasing throughput of microfluidic devices and automated data collection during bioimaging, the live cell imaging system can generate thousands of images every hour. For example, continuous running of the 1500-unit chip for one week generates 252,000 images. It

may take months to manually track the evolution of tissues and cell populations (e.g., the expression level of the fluorescently tagged biomolecules) at the single cell level. Using a customized MATLAB program, the massive data can be automatically sorted, formatted and analyzed. Traces showing the mobility, morphological features, and expression level of signaling molecules can be retrieved (shown in the video), which avoids human error and saves considerable amount of time.

Therefore, the methodology we demonstrate here show suitable protocols for performing high throughput biomedical experiments with automated cell and tissue manipulation, fluorescent imaging and data analysis. The on-off switching of membrane valves provides optimal liquid volume, time, and spatial resolution to reconstruct the ever-changing and complex environmental conditions for in vitro like an organ-on-chip. Even though the protocol is considerably more complex compared to the transitional biomedical approaches (e.g., manual procedures using 96-well plates), the presented protocol and platform are not restricted to studies on cell and tissue functions. The screening of combinatorial and sequential drug input may allow us to develop therapies for clinical applications.

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Disclosures:
The authors have nothing to disclose.

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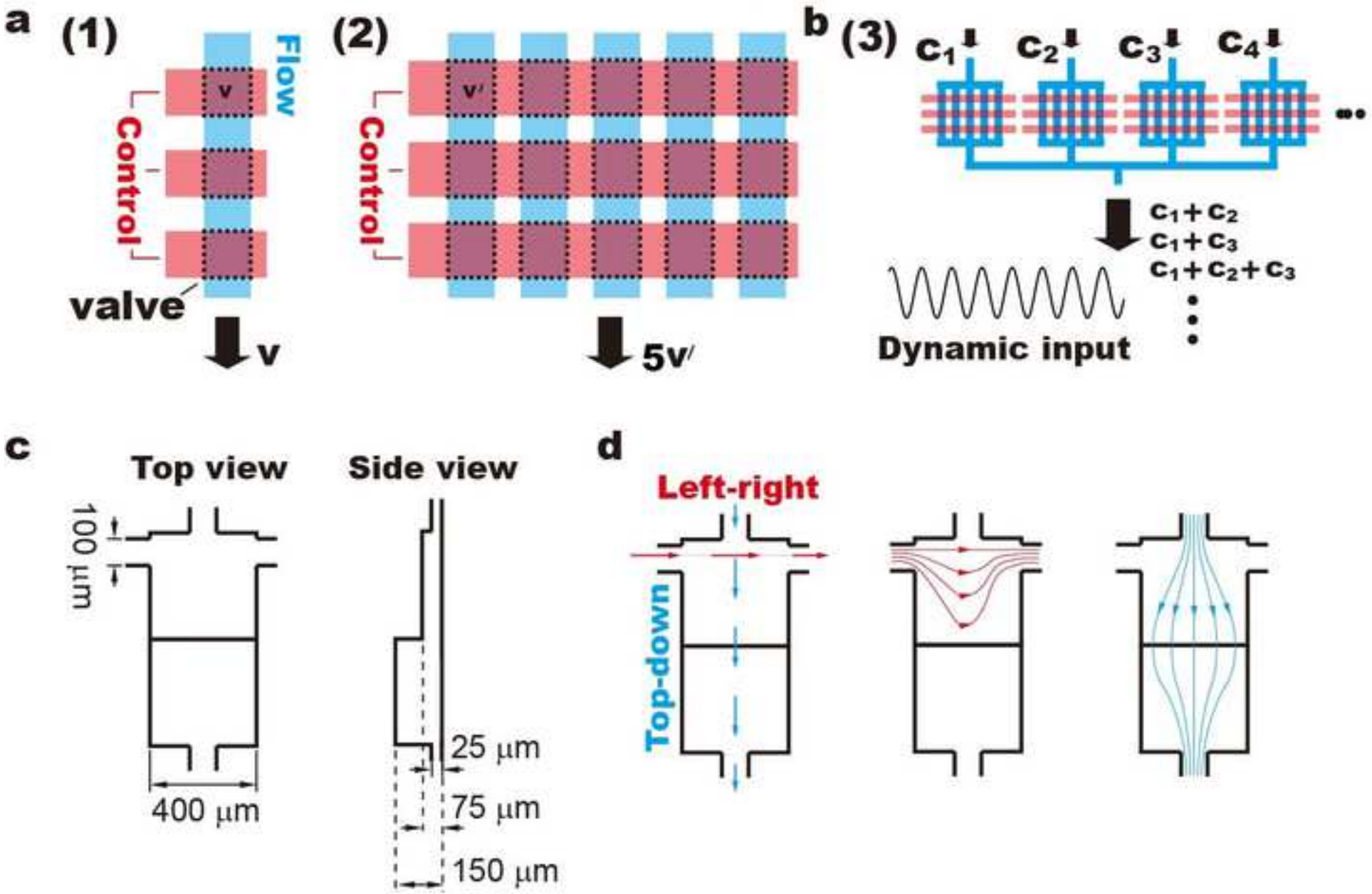

Figure 2

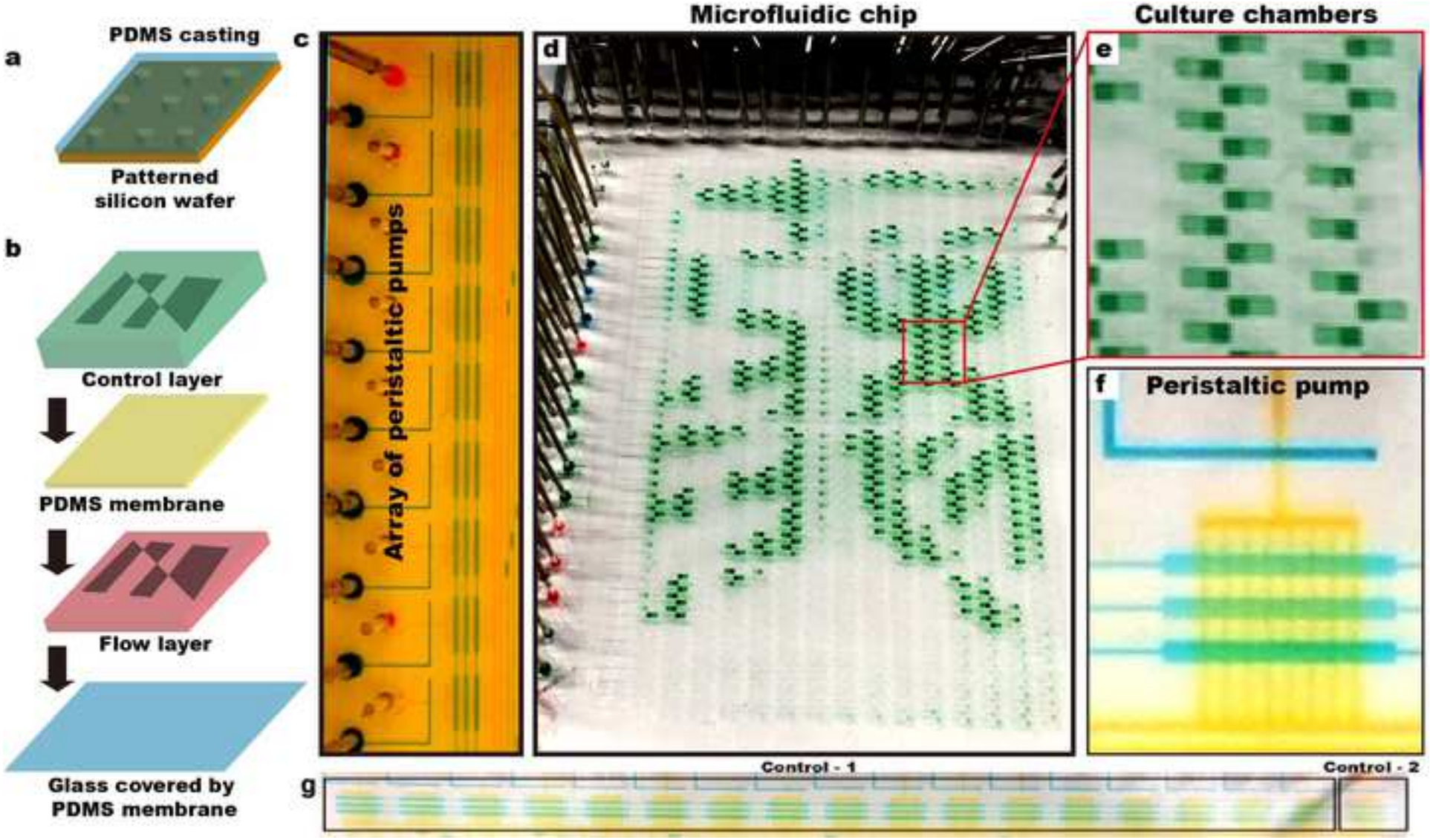

Figure 3

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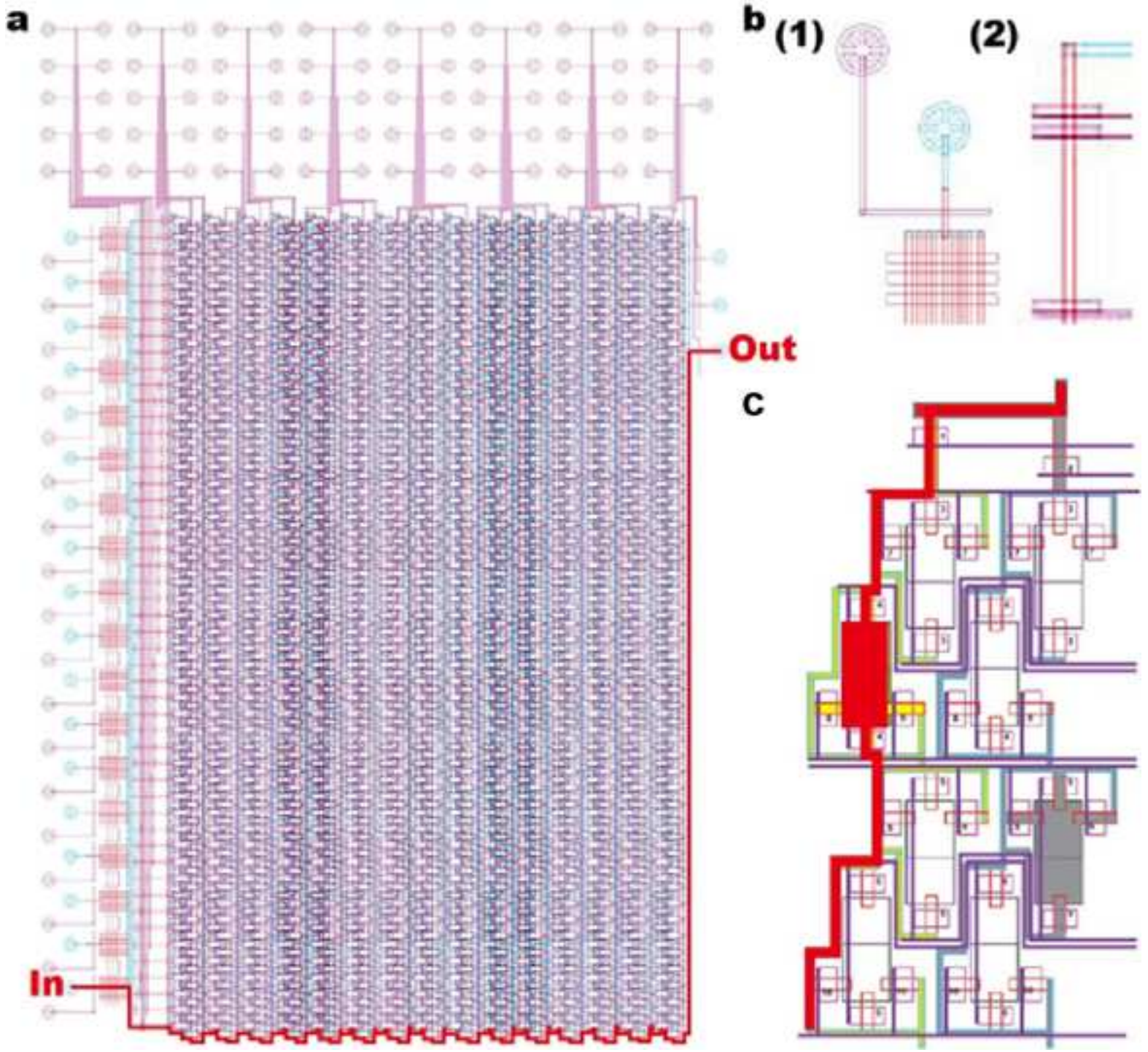

Figure 4

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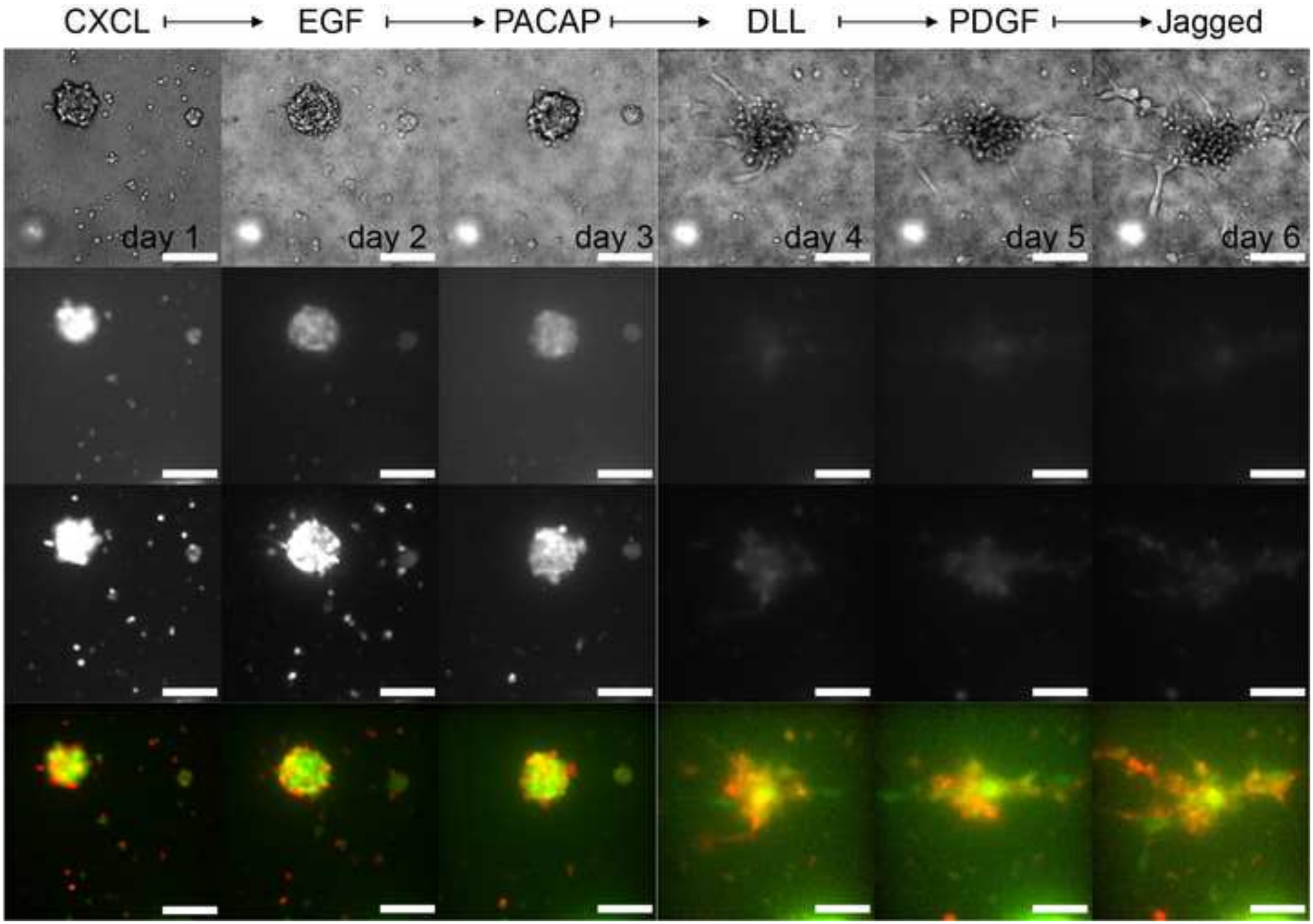

Figure 5

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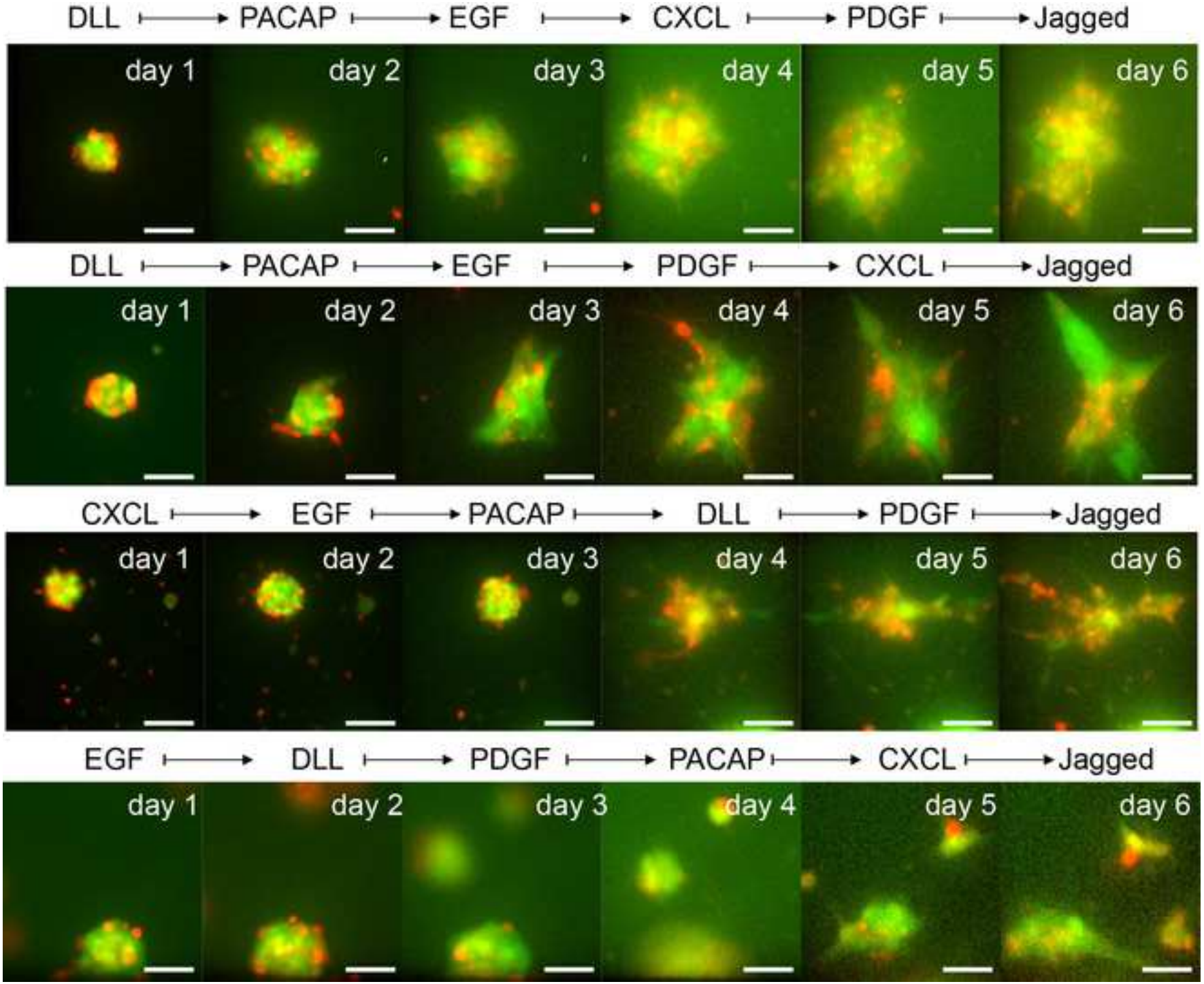

Table of Materials

Name of Mterial/Equipment	Company
2713 Loker Avenue West	Torrey pines scientific
AZ-50X	AZ Electronic Materials, Luxembourg
Chlorotrimethylsilane(TMCS) 92360-25mL	Sigma
CO2 Incubator HP151	Heal Force
Desktop Hole Puncher for PDMS chips WH-CF-14	Suzhou Wenhao Microfluidic Technology Co., Ltd.
DMEM(L-glutamine, High Glucose, henol Red)	Invitrogen
Electronic Balance UTP-313 Max:600g, e:0.1g, d:0.01g	Shanghai Hochoice Apparatus Manufacturer Co.,LTD.
FBS	Sigma
Fibronection 0.25 mg/mL	Millipore, Austria
Glutamax 100x	Gibco
Heating Incubator BGG-9240A	Shanghai bluepard instruments Co.,Ltd.
Nikon Model Eclipse Ti2-E	Nikon
Pen/Strep 10 Units/mL Penicillin 10 ug/mL Streptomycin	Invitrogen
Plasma cleaner PDC-002	Harrick Plasma
polydimethylsiloxane(PDMS)	Momentive
polylysine 0.01%	Sigma
Spin coater ARE-310	Awatori Rentaro
Spin coater TDZ5-WS	Cence
Spin coater WH-SC-01	Suzhou Wenhao Microfluidic Technology Co., Ltd.
SU-8 3025	MicroChem, Westborough, MA, USA
SU-8 3075	MicroChem, Westborough, MA, USA

Point-by-Point Response to Editor–
JoVE article JoVE61735R2

Editor

1. Changes to be made by the Author(s) regarding the written manuscript:

Comments:

1.1 Please revise the title to be Generation of Dynamical Environmental Conditions using a High-throughput Microfluidic Device and change this in the video title card as well

Response 1.1: We thank the editor for pointing out this issue. **The manuscript has been revised according to the editor's comments.**

1.2 Some additional revisions are needed on the written manuscript. Please see the comments in the attached manuscript.

Response 1.2: We thank the editor for pointing out this issue. The manuscript has been revised according to the editor's comments. As is shown in the manuscript line 5, line 7-10, line 25-27, line 30-37, line 78, 90, 98, 100, 103, 105, 113, 117, 135, and line 154, 156, 157.

1.3 Please ensure that the details of the protocol are the same as the narration in the video. There are some details in the video narration that should be included in the written protocol: Confluency of cells, DMEM media, etc.

Response 1.3: We thank the reviewer for pointing out this issue. The manuscript has been revised according to the editor's comments. As is shown in the manuscript line 159 and 162.

2. Changes to be made by the Author(s) regarding the video:

2.1 Please include a discrete representative results in the video after the protocol with a title card. Ideally, all figures in the video would appear in the written manuscript and vice versa. The video and the written manuscript should be reflections of each other.

Response 2.1: We thank the editor for pointing out this issue. The *figures were inserted at 2:37-2:40, 2:50-2:54, 2:59-3:20, 3:35-3:52, 4:30-4:33, 5:32-5:35, 7:56-7:59, 11:28-11:30* according to the editor's comments.

2.2 Video File Format & Quality:

2.2.1 The video resolution is too low. Please submit a video file with at least 1280x720 pixel resolution.

Response 2.2.1: We thank the editor for pointing out this issue. The video resolution has been revised according to the editor's comments.

2.2.2 Please ensure all audio level peaks are less than -6 dB. The average peaks should be around -9 dB. Some of the narration is a bit too loud in this respect.

Response 2.2.2: We thank the editor for pointing out this issue. The audio has been revised according to the editor's comments.