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Dr. Nandita Singh, Senior Science Editor
Journal of Visualized Experiments

Dear Dr. Nandita Singh,

On behalf of our co-authors, we would like to thank you for offering the opportunity for us to submit our microfluidic cancer-on-chip technology for consideration for publication in JoVE.

Primary and metastatic cancers develop as complex, heterogeneous ecosystems that continue to defy cure through conventional medical therapies. For example, cancers universally defeat treatment by chemotherapy through the emergence of drug resistant cells. Current therapies are developed through in vitro drug screening and tissue culture techniques that can detect initial drug sensitivity but are not designed to detect and measure drug resistance. Similarly, in vivo experiments in mice are designed to study sensitivity to therapies and not mechanisms of resistance because the animals succumb to the cancer.

We have designed an in vitro, self-contained, microfluidic cell culture system that reproducibly creates complex microenvironments (ecologies) that allows the direct observation of the development of drug resistance in real time. The device also allows quantitative analyses of motility and proliferation of heterogeneous cell populations and has the capability for downstream analyses of metabolites and single cells.

We have previously demonstrated using our microfluidic system the long-term co-culture of epithelial and mesenchymal PC3 prostate cancer cells (Lin *et al.*, *Convergent Science Physical Oncology*, 3(4): 045001 (2017)) as well as the emergence of drug-resistance polyploid giant cancer cells using the epithelial PC3 cell line (Lin *et al.*, *Clinical & Experimental Metastasis*, 36: 97 (2019)). We hope that you find the potential of this work as exciting as we do!

Sincerely,

Ke-Chih Lin
Ph.D. student, Princeton University

TITLE:

Generation of Heterogeneous Drug Gradients Across Cancer Populations on a Microfluidic Evolution Accelerator for Real-Time Observation

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KEYWORDS:

Microfabrication, cancer-on-a-chip, tumor microenvironment, chemotherapy gradient, resistance, cell migration.

SUMMARY:

We present a microfluidic cancer-on-chip model, the “Evolution Accelerator” technology, which provides a controllable platform for long-term real-time quantitative studies of cancer dynamics within well-defined environmental conditions at the single-cell level. This technology is expected to work as an in vitro model for fundamental research or pre-clinical drug development.

ABSTRACT:

Conventional cell culture remains the most frequently used preclinical model, despite its proven limited ability to predict clinical results in cancer. Microfluidic cancer-on-chip models have been proposed to bridge the gap between the oversimplified conventional 2D cultures and more complicated animal models, which have limited ability to produce reliable and reproducible quantitative results. Here, we present a microfluidic cancer-on-chip model that reproduces key components of a complex tumor microenvironment in a comprehensive manner, yet is simple enough to provide robust quantitative descriptions of cancer dynamics. This microfluidic cancer-on-chip model, the “Evolution Accelerator,” breaks down a large population of cancer cells into an interconnected array of tumor microenvironments while generating a heterogeneous chemotherapeutic stress landscape. The progression and the evolutionary dynamics of cancer in response to drug gradient can be monitored for weeks in real time, and numerous downstream experiments can be performed complementary to the time-lapse images taken through the course of the experiments.

INTRODUCTION:

Cancer has been increasingly recognized as a complex ecosystem that depends not only on the continued dysregulation of mutated cell populations but also on vital interactions between cancer cells and the host microenvironment. In this sense, cancer evolves on an adaptive landscape manifested by a combination of factors, including a heterogeneous tumor microenvironment and crosstalk with a variety of host cells, all of which contribute selective pressures for further genetic or epigenetic changes¹⁻³. In the context of solid tumors, uneven distribution of chemotherapeutics and other resource gradients contributes to their molecular heterogeneity and may play a role in the development of drug resistance, increased angiogenesis to particular tumor subpopulations, and even metastasis⁴⁻⁶. Conventional in vitro 2D cell culture studies, while possessing large-scale, convenient experimental capacity, provides mean-field, uniform, and fixed conditions, often lacking the precise spatial and temporal environmental control necessary to truly emulate in vivo tumor dynamics. Thus, there is a need for more representative ex vivo models to reproduce the tumor microenvironment prior to animal models in the drug development pipeline in order for a better prediction of cancer progression as well as responses to drugs within dynamical stress landscapes. Microfluidics have been proposed to bridge the gap between 2D cell culture studies and more complex in vivo animal studies that may not be able to support controllable quantitative studies⁷⁻⁹.

An ideal in vitro system to characterize cancer cell dynamics should possess the ability to generate a heterogeneous microenvironment to mimic the adaptive cellular responses that may take place in a tumor, as well as allow for the observation of these dynamics at a single-cell resolution. In this article, we describe a microfluidic cell culture platform, a PDMS-based device called the “Evolution Accelerator” (EA), that allows for parallel in vitro studies of cancer cell dynamics at cellular resolution with real-time data acquisition over the course of weeks, while stably maintaining gradients of stress across the culture landscape. The design of this platform is based on our previous work, in which the evolutionary dynamics of organisms in a metapopulation can be accelerated¹⁰⁻¹¹. Specifically, in a group of spatially separated populations that interact at some level, when exposed to a heterogeneous stress landscape, the most fit species can dominate in a local population faster compared with that of a large uniform population. The advantageous species then migrate to neighboring microhabitats in search of resources and space, and eventually dominate the entire population. As shown in **Figure 1**, the pattern of the microfluidic EA chip is composed of (i) a pair of serpentine channels that provide fresh media circulation and construct fixed boundary conditions for chemical diffusion, and (ii) the hexagonal cell culture region which consists 109 interconnected hexagonal and 24 half-hexagonal chambers in the center, resembling a honeycomb structure. The chip is 100 μm in depth. Media channels and cell culture region are connected with small slits (about 15 μm wide), which prevent direct media flow and the resulting shear stress across the cell culture area, yet still allow chemicals to diffuse through small slits and exchange nutrients, metabolic waste, etc. The generation of chemical gradients is demonstrated in **Figure 1B**, where one media channel contains 0.1 mM of fluorescein while the other channel is free of fluorescein. Cells are cultured on a gas permeable membrane, encapsulated by the microstructures through the positive back pressure on the membrane against the chip. The components of the device holder are illustrated

in **Figure 2**, and the experimental setup is illustrated in **Figure 3**, where the culture is maintained on an inverted microscope at 37 °C, with above 85% relative humidity, and conditioned under normoxia gas composition.

This system provides detailed observation of localized cellular interactions via brightfield and fluorescent channels and allows for spatially-resolved downstream assays such as immunofluorescence, Western blot, or mass spectrometry. We have previously demonstrated as a proof-of-principle of this microfluidic cancer-on-chip model on the long-term co-culture of epithelial and mesenchymal PC3 prostate cancer cells¹² as well as the emergence of drug-resistance polyploid giant cancer cells using the epithelial PC3 cell line¹³. While we present the application of this platform to understand the spatiotemporal dynamics of epithelial PC3 and mesenchymal prostate cancer cells under a stress gradient of docetaxel, the microfluidic system can be easily applied to any combination of cell lines and resource (i.e., drug, nutrient, oxygen) gradients.

PROTOCOL:

1. Fabrication of microfluidic device

1.1. Generate the desired microfluidic pattern using a layout design software (see **Supplemental Materials**).

1.2. Fabricate the photomask. See **Table of Materials** for more details.

1.2.1. Utilizing a laser writer, write the pattern on a soda-lime glass plate coated with 100 nm of Cr and 500 nm of photoresist AZ1518.

1.2.2. Develop the photoresist with developer AZ300MIF for 60 s.

1.2.3. Etch away the chromium without protection from the photoresist using Cr-7 Chromium Etchant.

1.2.4. Strip off residual photoresist using a photoresist stripper at 70 °C for 45 min.

1.3. Pattern the photoresist. See **Table of Materials** for more details.

1.3.1. Spin coat HMDS on silicon wafer at 4000 rpm for 40 s.

1.3.2. Spin coat photoresist AZ4330 on silicon wafer at 4000 rpm for 40 s.

1.3.3. Soft bake the silicon wafer at 95 °C for 60 s.

1.3.4. Utilize the mask aligner to expose UV to the silicon wafer.

133 1.3.5. Develop the photoresist with developer AZ300MIF for 4 min.
134
135 1.4. Perform DRIE etching. See **Table of Materials** for more details.
136
137 1.4.1. Dry-etch the wafer patterned with the photoresist using a Silicon Deep Reactive Ion Etching
138 (DRIE) system for 100 μm depth.
139
140 1.4.2. Strip off the photoresist with acetone and plasma etch with a plasma etcher.
141
142 1.5. Wafer oxidation and silanization. See **Table of Materials** for more details.
143
144 1.5.1. Perform thermal oxidation on the etched silicon wafer in a furnace at 1100 $^{\circ}\text{C}$ for 1 h.
145
146 1.5.2. Wait for the silicon wafer to cool down, and then place the wafer into a desiccator with a
147 few drops of trichloro-1H,1H,2H,2H-perfluorooctyl-silane (PFOTS) dripped in a small container
148 near the wafer.
149
150 1.5.3. Pump the pressure of the desiccator to 0.5 atm at room temperature for 60 min. The silicon
151 wafer mold should become hydrophobic after proper silanization, which can be tested by adding
152 several droplets of water onto the wafer.
153
154 1.6. Soft lithography. See **Table of Materials** for more details.
155
156 1.6.1. Mix pre-polymer and cross-linker (from polymethylsiloxane (PDMS) kit) at a 10:1 by weight
157 ratio.
158
159 1.6.2. Pour the mixed PDMS to create a 10 mm film in height onto the silanized silicon wafer
160 mold.
161
162 1.6.3. Degas the resulting PDMS-silicon wafer system in a desiccator for 30 min to 1 h.
163
164 1.6.4. Incubate the PDMS-silicon wafer in a 70 $^{\circ}\text{C}$ incubator overnight to cure the PDMS.
165
166 1.6.5. Once the PDMS is cured, peel the PDMS film off the silicon wafer carefully.
167
168 1.6.6. Using biopsy needles, punch through-holes at the inlet ports based on the location of the
169 pattern on the PDMS and employ a circular punch to cut chips 27 mm in diameter.
170
171 1.7. PDMS layer bonding.
172
173 1.7.1. Heat cure two stacks of 27 mm PDMS cylinders (without patterning), which will become
174 the reservoir layer and the capping layer of the device.
175

1.7.2. Cut out two 7 mm circles around the inlets on the reservoir layer, and utilize a biopsy needle to punch through-holes at the inlet ports on the capping layer.

1.7.3. Bond three stacks of PDMS (patterned stack, reservoir layer, capping layer) with oxygen plasma treatment.

1.7.4. With the biopsy needle, punch through-holes at the outlets and the center port of the device.

2. Media and cell line preparation

2.1. Prepare media for culturing PC3 cell lines, including PC3-EMT and PC3-EPI: mix RPMI 1640 medium with 10% fetal bovine serum (FBS) and 1x antibiotic-antimycotic. Generate cell lines as described previously¹⁴.

NOTE: PC3 cell lines are maintained in the above media in humidified incubators at 37 °C with 5% CO₂. Split cells and subculture every 3 days, before they reach 100% confluency.

2.2. Transfect cell lines with cytoplasmic-labeled fluorescent markers for better visualization, as described previously¹², such that PC3-EMT expresses cytoplasmic GFP and PC3-EPI expresses cytoplasmic mCherry. Note that the technology is also compatible with any fluorescent-labeled cells as well as brightfield imaging of unlabeled cells.

3. Experimental setup

3.1. Fabrication of metal plate holder

3.1.1. Fabricate a plate holder that is sufficient to hold simultaneous gas-permeable culture dishes for parallel experiments by machining or 3D printing. The 3D CAD file ("3-well plate.FCStd") can be found on GitHub as a reference (<https://github.com/kechihi/3-well-plate>).

3.1.2. Unscrew the components of the holder (**Figure 2**) with a screwdriver. Disinfect the components via UV exposure for at least 1 h and leave in a sterile environment.

NOTE: The holder is designed to provide substantial conditions for thermal equilibrium and ideal gas compositions, with gas channel inlets that allow for airflow. More details can be found in our previous work¹².

3.1.3. Prepare up to three gas-permeable culture dishes, of which the cell culture membrane is relatively flexible.

3.2. Cell line seeding

3.2.1. 24 hours before the start of the experiment, harvest PC3-EPI and PC3-EMT cells by trypsinization for 5 min. Add prewarmed culture media, then centrifuge at 150 x *g* for 3 min and discard the supernatant.

3.2.2. Count cells of each PC3-EPI and PC3-EMT using a hemocytometer and isolate a total of 2.5×10^4 of each cell type for each gas-permeable culture dish.

3.2.3. Mix and resuspend the two cell types in 2 mL of culture media and seed the cells into each of the gas-permeable culture dish.

3.2.4. Leave the entire plate holder in the incubator overnight for cells to attach.

3.2.5. Disinfect the PDMS devices via UV exposure for at least 1 h and leave in a sterile environment.

3.3. Setting up thermal control unit and gas supply system

3.3.1. As shown in **Figure 3**, set up a gas supply system that consists of both CO₂ and O₂ control units, a gas pump, a gas mixing chamber, a humidifier or bubbler, and three separate sets of gas valves and pressure gauges. More details can be found in our previous work¹².

3.3.2. Set up the CO₂ and O₂ control units such that they adjust the mixing rate of the gas from CO₂ and N₂ tanks and the O₂ source. Alternatively, any gas supply system that provides gas under normoxia condition would work.

3.3.3. Make sure the gas that is combined in the mixing chamber and humidified by a bubbler such that the relative humidity is increased up to 85% (as read out from a relative humidity monitor) and that the gas leads to three independent gas valves with pressure gauges in order to control and monitor gas flow rate in the plate holder.

3.3.4. Place the entire plate holder in an on-stage incubation thermal control unit with separate heating subunits for the lid and the bottom plate, with all units set at 37 °C. See **Table of Materials** for more details.

3.4. Installation of microfluidic device. See **Table of Materials** for more details.

3.4.1. Identify that the detailed components of the 3-well plate holder are in order, as in **Figure 2**. Each of the three wells are identical and independent, with a pair of gas channels for each well. Every well possesses a gas-permeable culture dish holder, a PDMS chip holder, a glass window holder, and a pair of 35 mm glass windows. These components can be assembled using the fitted screws.

NOTE: The glass windows ensure that there is a thermally-isolated space between the gas-permeable culture membrane and the well such that no water condensation occurs due to

temperature differences at the interface. Note that the 3 wells are independent, and 3 experiments can be done separately.

3.4.2. Pre-warm culture medium at 37 °C and degas in a vacuum chamber for 20 min.

3.4.3. Treat the PDMS chips using an oxygen plasma system for 30 s in order to maintain hydrophilicity.

3.4.4. Set up the syringe system. Load two syringes slowly with growth media and other two syringes with desired reagent of interest (media, media with drug, etc.). Connect each individual syringe to a 50 cm tubing (0.020" x 0.060"OD) by a 23 G dispensing needle into one hollow steel pin. Insert a hollow steel pin into the other end of the tubing.

3.4.5. Prime the tubing and insert the steel pin into each PDMS chip through the capping layer. Fill up the reservoir layer and wet the PDMS pattern layer pattern with media. The reservoir layer works as an on-chip bubble trap to prevent air bubbles from getting into the microfluidic pattern.

3.4.6. Load a 1 mL syringe with culture media. Connect the syringe to a 5 cm tubing (0.020" x 0.060"OD) by a 23 G dispensing needle into one hollow steel pin. Insert a hollow steel pin into the other end of the tubing and prime the tubing.

3.4.7. Insert the hollow steel pin into the center hole of the chip, where excessive media can be extracted out from the chip later during the chip sealing process.

3.4.8. As each PDMS device requires four 10 mL syringes loaded onto a syringe pump, load two 10 mL syringes per chip in the forward deck. Place the two other syringes in the withdraw deck of the syringe system.

3.4.9. Place chip directly on top of the gas-permeable culture membranes (with cells already adhered to the membranes). In order to avoid entrapping microbubbles in the microfluidic pattern, dispense 1 mL of prewarmed and degassed media into the 35 mm gas-permeable culture dish before assembly, and then make sure that the chip approaches the liquid surface with a 15-degree tilt angle.

3.4.10. Clamp the gas-permeable culture dish and the well in the gas-permeable culture dish holder for each chip, with the PDMS chip holder pushing the PDMS device downward.

3.4.11. Tape a sheet of a sealer on top of the PDMS device and clamp with the PDMS chip holder in order to prevent the chip from drying out.

3.4.12. Set media flow rate around the array to be 20 μ L/h.

3.4.13. Set the entire plate in the on-stage incubator on the motorized stage of an inverted microscope. Connect the gas supply system to the gas channels and pressurizing the gas-

permeable culture membrane against the installed PDMS chip to ensure sealing of device. Maintain gauge pressure at 0.2 psi (1.4×10^4 Pa).

3.4.14. Slowly extract excessive media in the chip using the 1-mL syringe from the center hole. Observe the chip under the microscope while extracting media and then stop extracting when the chip is sealed. Chip sealing should be obvious since a part of the cells would be crushed by the micro-structures.

3.4.15. Connect the temperature sensing unit of the on-stage incubator to the 3-well plate and set to 37 °C.

4. Single-cell time-lapse imaging

4.1. Set up imaging software utilizing an inverted microscope to time-lapse image acquisition. Note that an inverted fluorescent microscope with fully motorized x-y stage, focus knob, shutter, and filter cubes is required for an EA experiment.

4.2. Configure software to acquire images across two channels at 10x magnification for each chip with automatic image-stitching after one round of autofocus. Be aware that automatic correction systems like Perfect Focus do not guarantee satisfying image quality. It is more recommended to generate a customized focus surface for long-term image acquisition based on either autofocus or manual focus across the chip.

4.3. Take images every hour and leave experiment running on the time scale of weeks. Monitor image quality on a daily basis and update the focus surface if necessary.

4.4. Prepare the PDMS chip and gas-permeable culture dish for subsequent immunofluorescent analysis, as described previously¹³.

5. Image processing and analysis

5.1. Post-experimental processing

5.1.1. Convert images into TIFF format for image processing and measurements utilizing Fiji/ImageJ.

5.1.2. Compress TIFF files for ease of further image processing.

5.2. Fiji/ImageJ analysis

5.2.1. To identify cells, perform Background Subtraction and Particle Analysis to identify fluorescent bright-spots for cell location detection and automatic cell counting.

5.2.2. Utilize plugins (Manual Tracking, Chemotaxis, Migration Tool, Trackmate) to analyze cell motility and migration.

REPRESENTATIVE RESULTS:

Validation of optimum cell growth on chip

A major goal of the experiment platform is to reproduce key components and interactions in a complex tumor microenvironment in a comprehensive manner, yet simple enough to provide quantitative, reliable and reproducible data. This goal can only be achieved if we have full control of the physical and biochemical environmental factors. We must either exclude the undesired factors or figure out a way to incorporate the uncontrollable factors into the quantitative model to correctly interpret the population behaviors.

The first test is to ensure optimum cell growth on the device. The cell proliferation rate on the device needs to be identical or comparable to their growth profile in conventional cell culture. As a proof of principle, human prostate cancer cell line PC3 cells were cultured in the PDMS chip without the presence of external stress. These cells were fluorescently-labeled in the cytoplasm so we could observe and quantify the population of cells using inverted fluorescent microscopy. Cells confluence, defined as the area that covered by cells, was measured at each microhabitat at different time frames given a selected intensity threshold on the fluorescent images using Fiji¹⁵. The growth curves of cells at each chosen position are plotted in **Figure 4**. The chosen positions are scattered across the chip, from the region near the apex of media inflow to the apex close to media outlets.

The growth curves in **Figure 4B** are well-aligned, suggesting the identity of the cell proliferation profile as a function of position across the chip. The initial slope of the curves reveals the proliferation rate. The doubling time for cells in the device is around 24 hours as shown in **Figure 4B** from time 0 to 24 hours, which is the same as the doubling time in conventional cultureware¹³. Therefore, we conclude that without the presence of an external source of stress, the physical and biochemical factors are uniformly distributed, leading to the homogeneous and optimized cell growth from time 50 to 100 hours.

We further considered the ability to quantify cell dynamics between subpopulations in a microenvironment. GFP-expressing PC3-EMT and the mCherry-expressing PC3-EPI cell lines were co-cultured in the EA as shown in **Figure 5**. Cell confluence, defined as the percentage of area covered by the cells, is measured as the indication of the size of population. Starting from 40% confluence, the co-culture grew fully confluent within 3 days. Interestingly, we may observe the progression of each phenotype in the microenvironment. While the growth curve of total confluence is in the form of logistic growth model, the population of PC3-EMT continued to expand and PC3-EPI was suppressed in the asymptotic phase.

Demonstration of cell motility assay in a mixed population

Cell motility is an important phenotypic behavior. The motility distribution of a population can provide information about the physical interaction of cells, the mechanical properties of local microenvironment, and can sometimes be analyzed as an indication of epigenetic variation of

cells. Given that our improved cell culture platform allows nearly continuous imaging, the advantage of having fine temporal resolution maximizes the potential of single cell tracking in a heterogeneous population.

We use the plugin TrackMate¹⁶ in Fiji (<http://fiji.sc>) with customized macro to implement and automate the tracking process. TrackMate allows user to implement and customize a tracking algorithm using a scripting language from the Fiji Script Editor. The tracking process is divided in a series of steps: the segmentation, filtering, and particle-linking processes.

As shown in **Figure 6B**, we performed a “wound healing assay” as a demonstration to study cell collective migration and cell-cell interaction. PC3-EPI and PC3-EMT cells were densely seeded in the center of the device and were allowed to freely migrate toward empty region. The normalized histogram of the speed of both phenotypes is plotted in **Figure 6C**. The velocity of PC3-EMT was significantly higher than PC3-EPI by a factor of 1.8x, which is consistent with the fact that the mesenchymal phenotype serves as a component of cutaneous wound healing with exceptional ability to migrate as opposed to the comparably stationary epithelial phenotype.

Further, in order to observe the progression of cell motility in a stress-free environment, we performed a long-term co-culture of PC3-EPI and PC3-EMT with uniform initial seeding density and tracked the distribution of cell velocity varying with time. As shown in **Figure 7A,B**, after 6 days of culture, as cells reached higher confluence, the distributions of cell speed for both phenotypes leaned toward the left tails.

We may compare the ratio of number of PC3-EPI to PC3-EMT in each class interval. As shown in **Figure 7C**, regardless of the days of culture or density of cells, PC3-EMT dominated the high-speed region while PC3-EPI occupied the low speed zone. Although the overall velocity decreased significantly for both phenotypes, PC3-EMT remained motile and was affected less by the crowdedness of the microenvironment as shown in **Figure 7D**. The average speed of PC3-EPI dropped around 40% while that of PC3-EMT only dropped 14%.

There are several factors that can affect the distribution of cell velocity and the average speed, such as the crowdedness of the population. Continuous cell tracking and monitoring over all subpopulation is essential to understand the progression of phenotypic behavior. For example, if we would like to quantify the level of the mesenchymal status of an unlabeled subpopulation, it would be more informative to acquire not only the distribution of the physical quantity (e.g., velocity) of the interested subpopulation as a function of time, but also that of all the rest of the coexisting subpopulation of cells.

FIGURE AND TABLE LEGENDS:

Figure 1. The syringe-pump-driven microfluidic EA chip. (A) The microfluidic device has two separate medium inlets, two outlets, and one central port for cell seeding or extraction of excessive medium. Note that between the medium inlets and the cell culture arrays, a pair of serpentine channels that allow the medium to equilibrate with the atmosphere passed below the

gas-permeable membrane. **(B)** The generation of gradient. The syringes pump the medium with ~ 0.1 mM of fluorescein and no fluorescein respectively into the chip through the 2 medium inlets on the right, extracted out from the chip at the 2 medium outlets on the left. The concentration of fluorescein is proportional to the intensity of fluorescence signal, and is profiled near the medium inlets/outlets a and near the center b. Figure reproduced with permission from Lin et al. 2017¹².

Figure 2. The components of the customized 3 well sample plate. (a) the main body of 3 well plate; (b) a pair of gas channels that allow conditioned atmosphere to be pumped in and vented out through the septa at the entrance of the gas channels; (c) an O-ring designed to seal the space between the well plate and the gas-permeable culture dish; (d) the 35 mm diameter gas-permeable culture dish; (e) the dish holder; (f) the PDMS device; (g) the PDMS chip holders; (h) the double layer 35 mm glass windows designed to maintain thermal isolation and prevent water condensation; (i) glass window holder; (j) heating pads; (k) temperature sensor; (l) the Adhesive sealer that keeps the chip from drying out. Figure reproduced with permission from Lin et al. 2017¹².

Figure 3. The schematic figure of the experimental setup. The setup of the experiment, including the gas supply system, gas channels connection, the medium supply connection and the imaging system. Figure reproduced with permission from Lin et al. 2017¹².

Figure 4. Growth curves of PC3 in the EA without stress. PC3 were cultured in EA without the presence of external stress. **(A)** Pattern of EA with position labels. **(B)** Growth curves of PC3 at the chosen positions in terms of cell confluence.

Figure 5. The co-culture of PC3-EPI and PC3-EMT in the EA without stress. PC3-EPI (red) and PC3-EMT (green) were co-cultured in the EA without the presence of external stress. **(A)** Overlay of two fluorescent channels at $t = 0$ h. **(B)** Fluorescent image taken at $t = 95$ h. **(C)** Growth curves in terms of cell confluence. Cell confluence of each cell type was measured on the entire chip. Each data point represents the average cell confluence at 3 consecutive time points taken at 15-min intervals. The error bars represent the standard deviation of cell confluence at the 3 consecutive time points.

Figure 6. Cell motility assay in a mixed population. The demonstration of single cell tracking performance in a mixed population. **(A)** A demonstration of how cells are detected by Laplacian of Gaussian (LoG) segmentation. The circles indicate the location of cells detected by the LoG algorithm. The detected cells are linked from frame to frame based on the linear assignment problem to quantify cell motility. **(B)** The “wound healing assay” of PC3-EPI and PC3-EMT co-culture experiment. Cells were seeded locally up to 100% confluence with a clear boundary at the center. After the installation of the EA chip, cells were observed to migrate through the microhabitat array as indicated by the white arrows. **(C)** Normalized histogram of cell motility. PC3-EMT is faster than PC3-EPI by a factor of 1.8x. The error bars represent the standard deviation of 5 experiment samples.

Figure 7. The motility assay of a long-term co-culture of PC3-EPI and PC3-EMT. A long-term co-culture of PC3-EPI and PC3-EMT with uniform initial seeding density. The distribution of cell velocity varies with time. **(A)** Normalized distribution of cell speed on day 2. **(B)** The normalized distribution of cell speed on day 6. **(C)** Ratio of number of PC3-EPI to PC3-EMT in each class interval. **(D)** Average speed of PC3-EPI and PC3-EMT as a function of time.

DISCUSSION:

Conventional cell culture was developed almost a century ago and remains the most frequently used preclinical model in biomedical research, despite its proven limited ability to predict clinical results in cancer¹⁷. Animal models offer the highest physiological relevance and reasonable genetic similarity to humans, but have long been acknowledged to have significant limitations in predicting human outcomes¹⁸. Among all the existing preclinical models, microfluidic cancer-on-chip models appear to be the most promising candidate to satisfy the unmet need in cancer research^{9,19,20}. However, current cancer-on-chip models have yet to offer a platform that is able to reproduce key components and interactions to mimic a tumor microenvironment in a comprehensive manner, yet simple enough to provide reliable and reproducible quantitative data. Our representative data demonstrate that our microfluidic cell culture technology provides stable physical and biochemical conditions for long-term cell culture in heterogeneous micro-environments. This multifunctional platform allows the sophisticated adjustment of a time--dependent chemical gradient as well as ambient gas composition. The platform is portable and compatible to most of the fluorescent inverted microscopes for high--resolution real-time imaging, which offers multidimensional information of cells as a function of time, including cell morphology, population dynamics, cell motility and cell migration on a single-cell level.

Compared to our previous work¹¹, we note that this pressure-sealing packaging and assembly method for our microfluidic device enables numerous downstream experiment capacities (e.g., FACS, local metabolite extraction, sub-clonal populations expansion, IF, DNA/RNA FISH, etc.) complementary to the time-lapse images taken in the course of the experiments. This capability of removing cells from specific locations in a heterogeneous culture is a major advance compared to earlier devices, where the sealing surface to a chip had to be removed before one could access the culture¹¹. Further, even in that case, the act of removing the cover significantly disturbed the culture so that local testing of cells on a single to few habitat levels was not possible. Such localized extraction was possible in our case due to the soft "resealable" nature of the flexible gas-permeable membrane.

There are several critical steps in the presented protocol. First of all, to initiate a long-term experiment with stable fluidic dynamics, microbubbles need to be avoided as much as possible. Therefore, it is important to prewarm and degas the culture medium (step 3.4.2) before loading the media into the syringes. The manipulation of media loading and dispensing should be done slowly and gently to prevent bubble formation. The PDMS pattern should be treated with oxygen plasma (step 3.4.3) before connecting to the culture media supply system. Oxygen plasma treatment ensures the hydrophilicity of the PDMS surface and significantly reduces the possibility of microbubble entrapping. In step 3.4.7., when placing the chip on top of the cell culture membrane, the PDMS chip should approach the liquid surface with a 15-degree tilt angle to get

rid of the bubbles (if any) on the liquid surface inside the culture dish. Secondly, the pressure-sealing method requires a stable gas supply system that pressurizes the cell culture membrane. The pressure should be set between 0.2 to 0.6 psi. Pressure lower than 0.2 psi is insufficient for chip sealing. Above 1.2 psi, a significant amount of gas would permeate through the membrane and generate bubbles within the microfluidic pattern. We note that if the components of the metal 3-well plate are not properly assembled (including the plate body, the gas-permeable culture dish holder, a PDMS chip holder, a glass window holder, O-rings, and a pair of 35 mm glass windows), one would identify gas leakage as the pressure reading would be irresponsive to the increase of the gas flow. The leakage issue can be resolved simply by doing a leakage test and then reassembling the components.

The major form of data acquired with the utilization of the EA technology is through imaging. As mentioned previously, the EA technology can be installed on any inverted microscope. However, to fully exploit the advantages of the system, it is highly recommended to install the system on an inverted fluorescent microscope equipped with fully motorized x-y stage, motorized focus knob, motorized shutter, and motorized filter cube. Also, be aware that Perfect Focus might not guarantee satisfying image quality. We suggest using an imaging software that allows multi-dimensional image acquisition and customized imaging script. Keeping the images well-focused could be challenging without a periodic (every 24 hours or 48 hours) autofocus command, which generated a customized focus surface during the image acquisition cycle.

The presented microfluidic EA technology has been implemented to study the adaptation and evolution dynamics in prostate cancer cell metapopulations under a chemotherapeutic stress landscape¹². We have also demonstrated the emergence of drug-resistance polyploid giant cancer cells in this tumor-like heterogeneous micro-ecology, showing that environmental heterogeneity combined with cell migration within tumor population would cause amplification on the emergence of resistant phenotypes¹³. With the ability to control and monitor the behaviors of mixed tumor cell populations under well-controlled stress gradients, our microfluidic EA technology may also serve as a platform to study regulatory mechanisms and the phenotypic transformation involved in EMT in the context of cancer therapeutic strategies²¹. Last but not least, the presented pressure-sealing packaging method can also be implemented in other microfluidic systems that require the sophisticated adjustment of ambient gas composition as well as the downstream experiment capacities. For instance, the same packaging method was used to incorporate different microfluidic pattern to study how bacterial population waves propagate through physical barriers²².

In summary, collective population dynamics are qualitatively different from single-cell behaviors. The microfluidic EA technology allows the quantitative study of the evolutionary dynamics and phenotypic behaviors individually and collectively as a function of time. This technology may present a more physiologically relevant in vitro model for cancer research, and potentially for preclinical drug development and screening.

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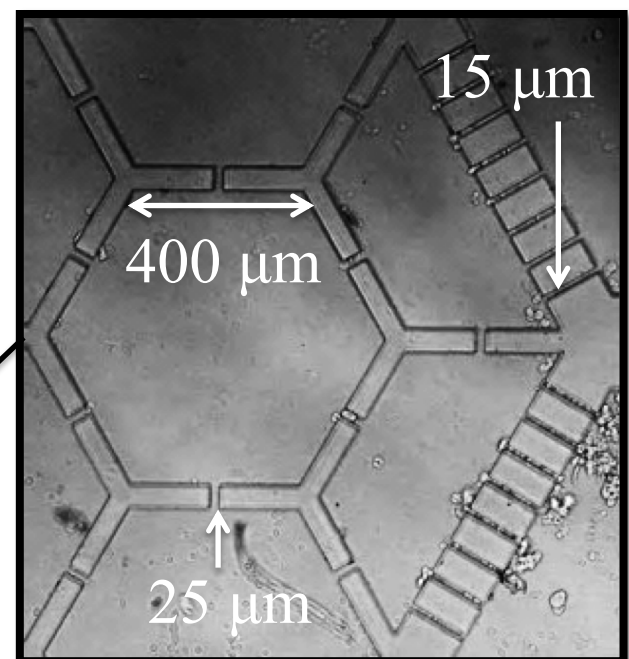
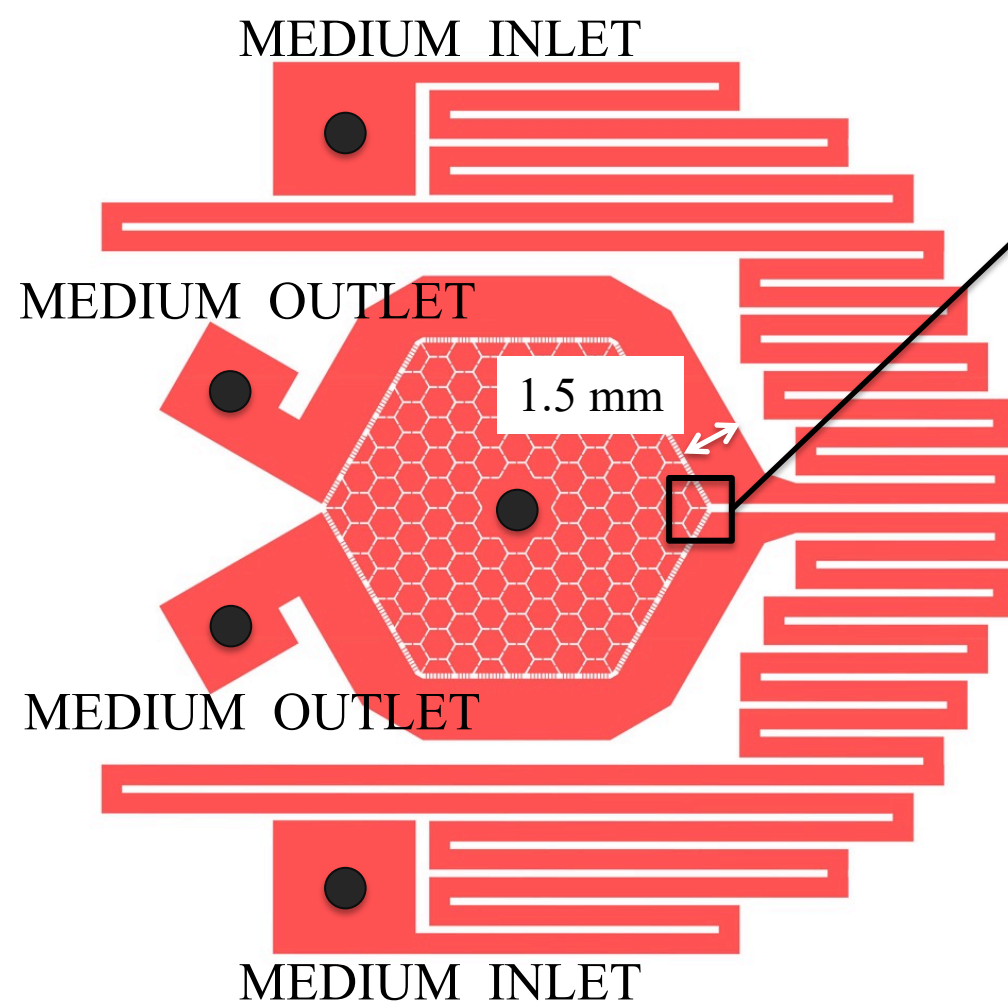
DISCLOSURES:

No conflicts of interest declared.

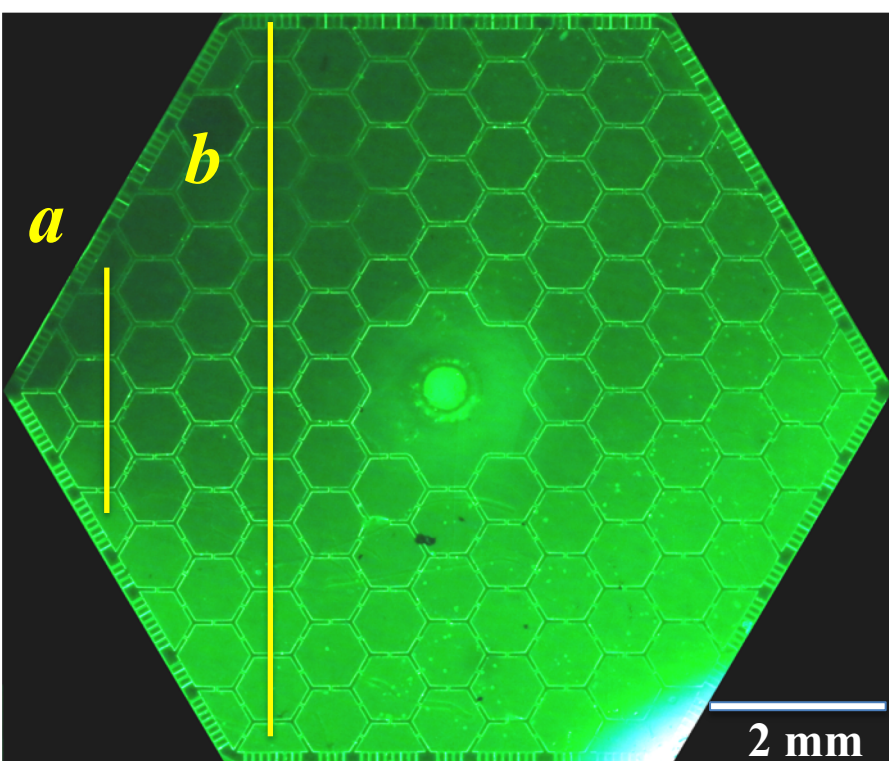
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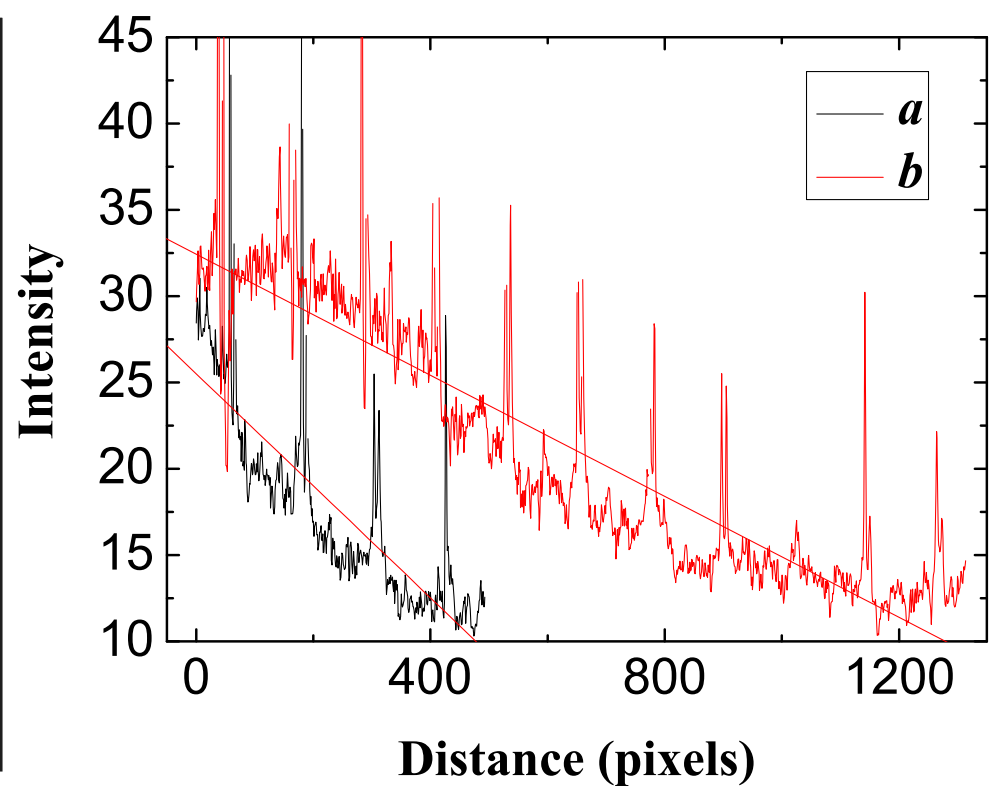
A

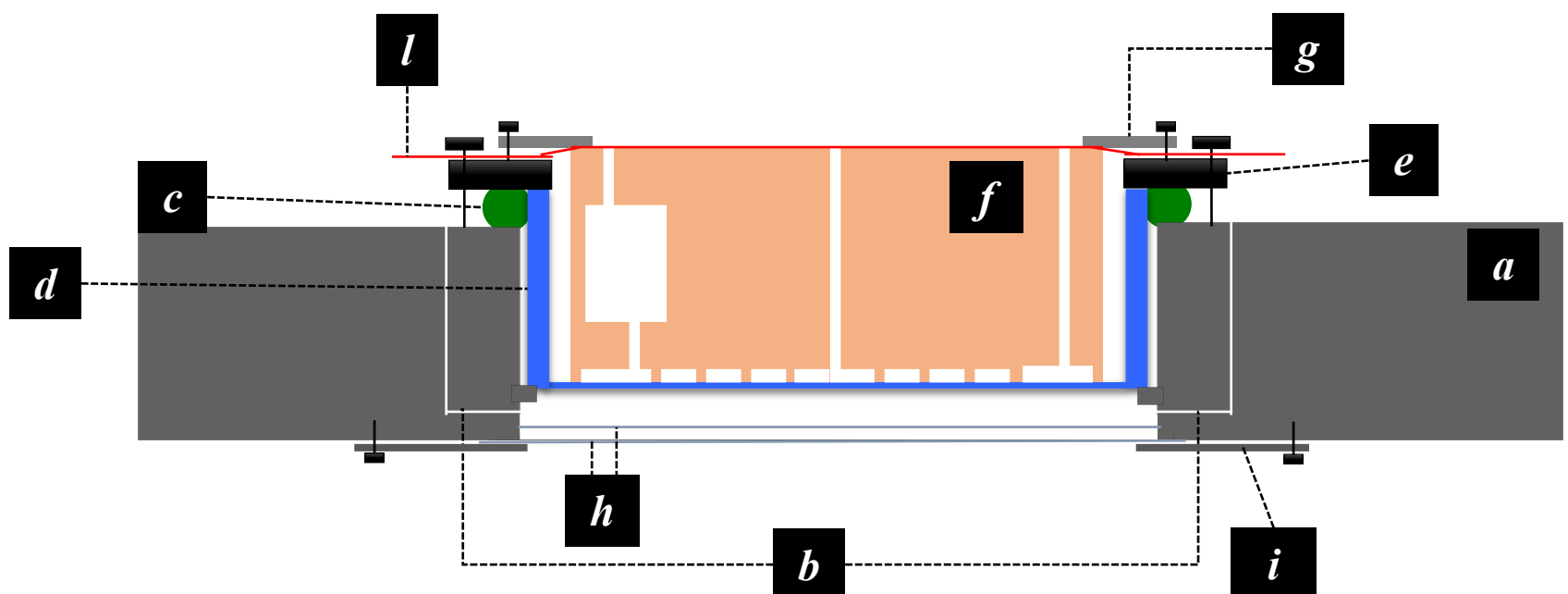
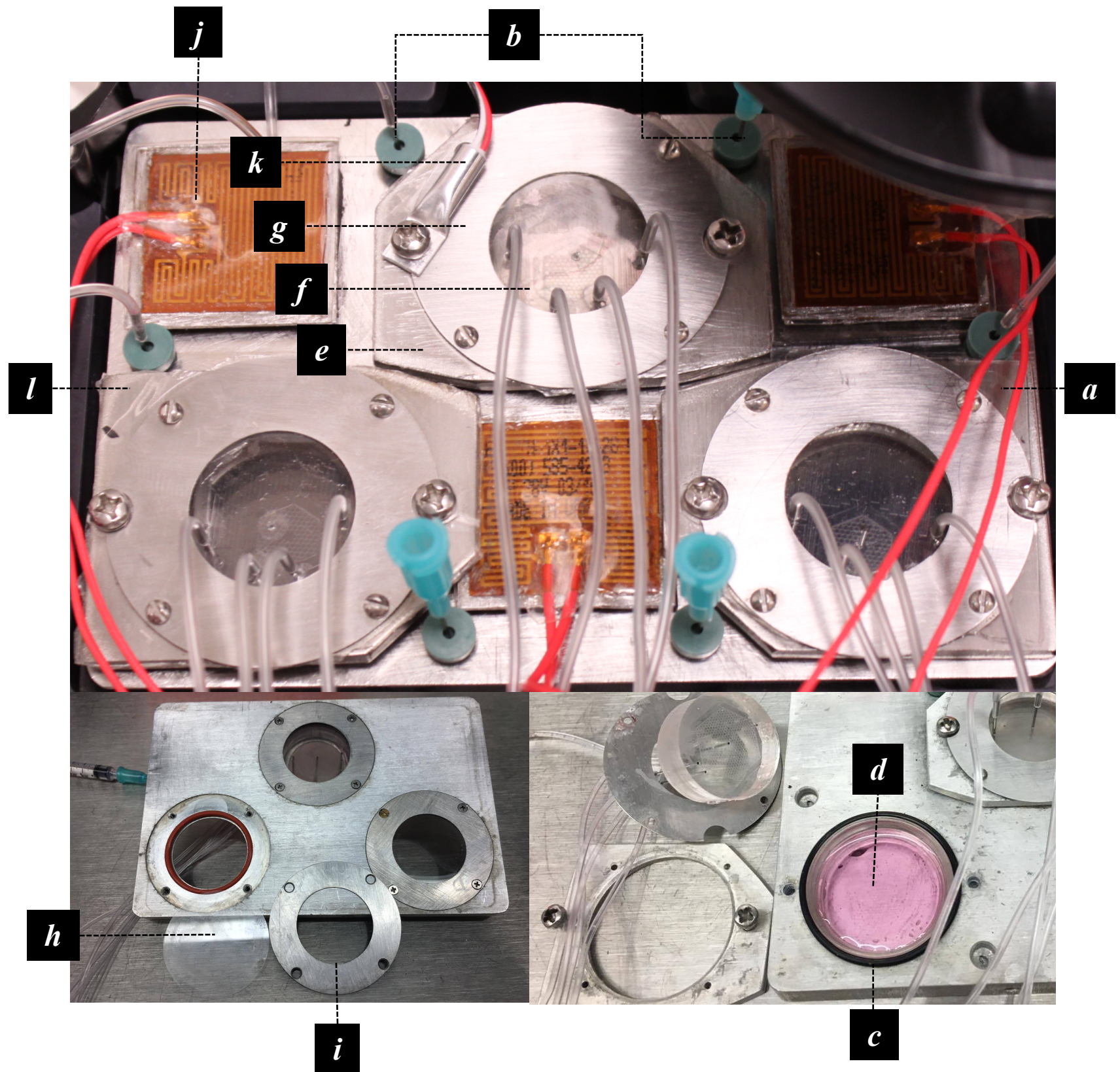
THE ASSEMBLED
EVOLUTION ACCELERATOR

B

$$a: Y = (25.51 \pm 0.30) + (-3.25\text{E-}2 \pm 1.06\text{E-}3) X$$

$$b: Y = (32.43 \pm 0.18) + (-1.75\text{E-}2 \pm 2.31\text{E-}4) X$$





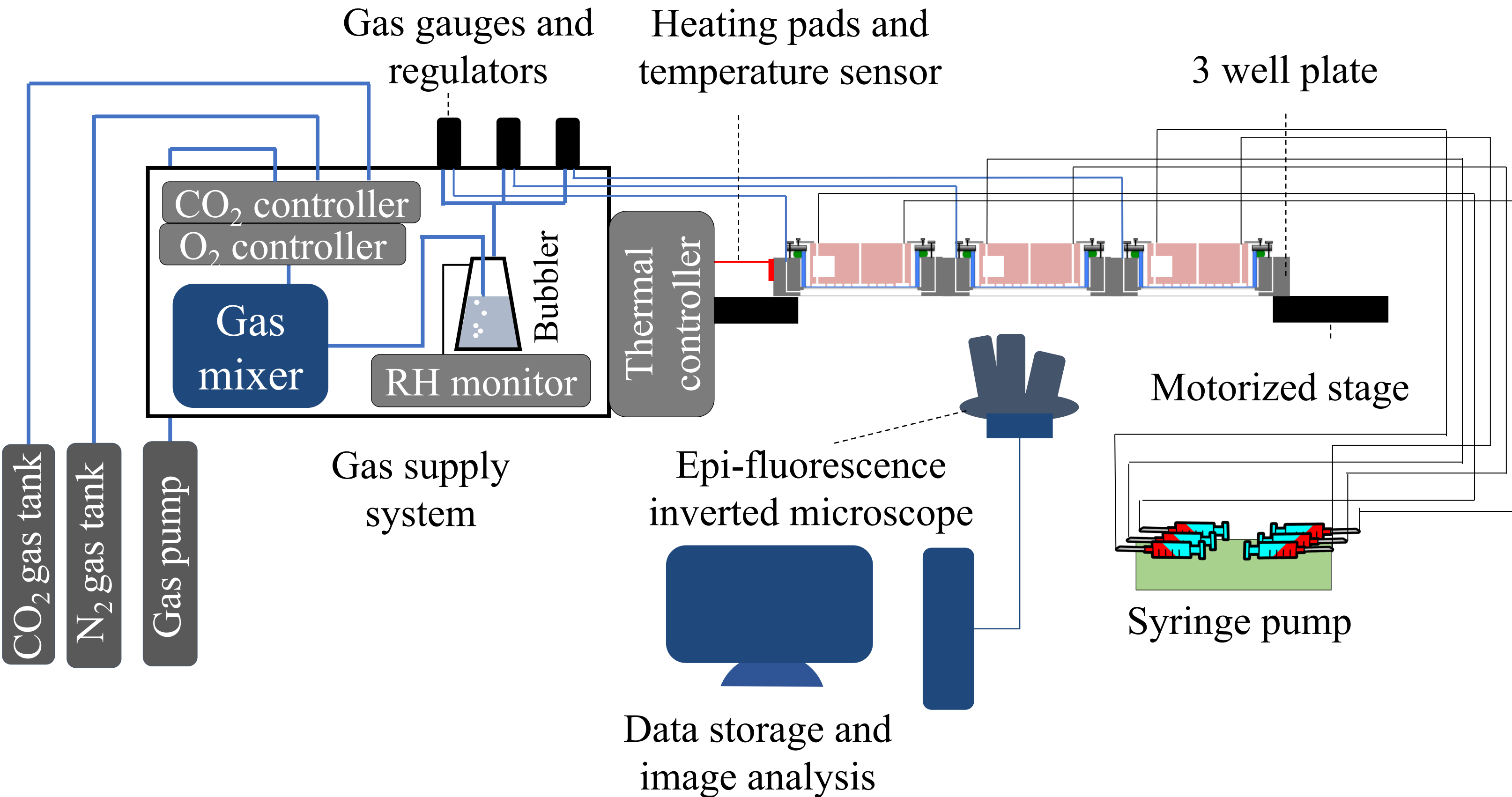
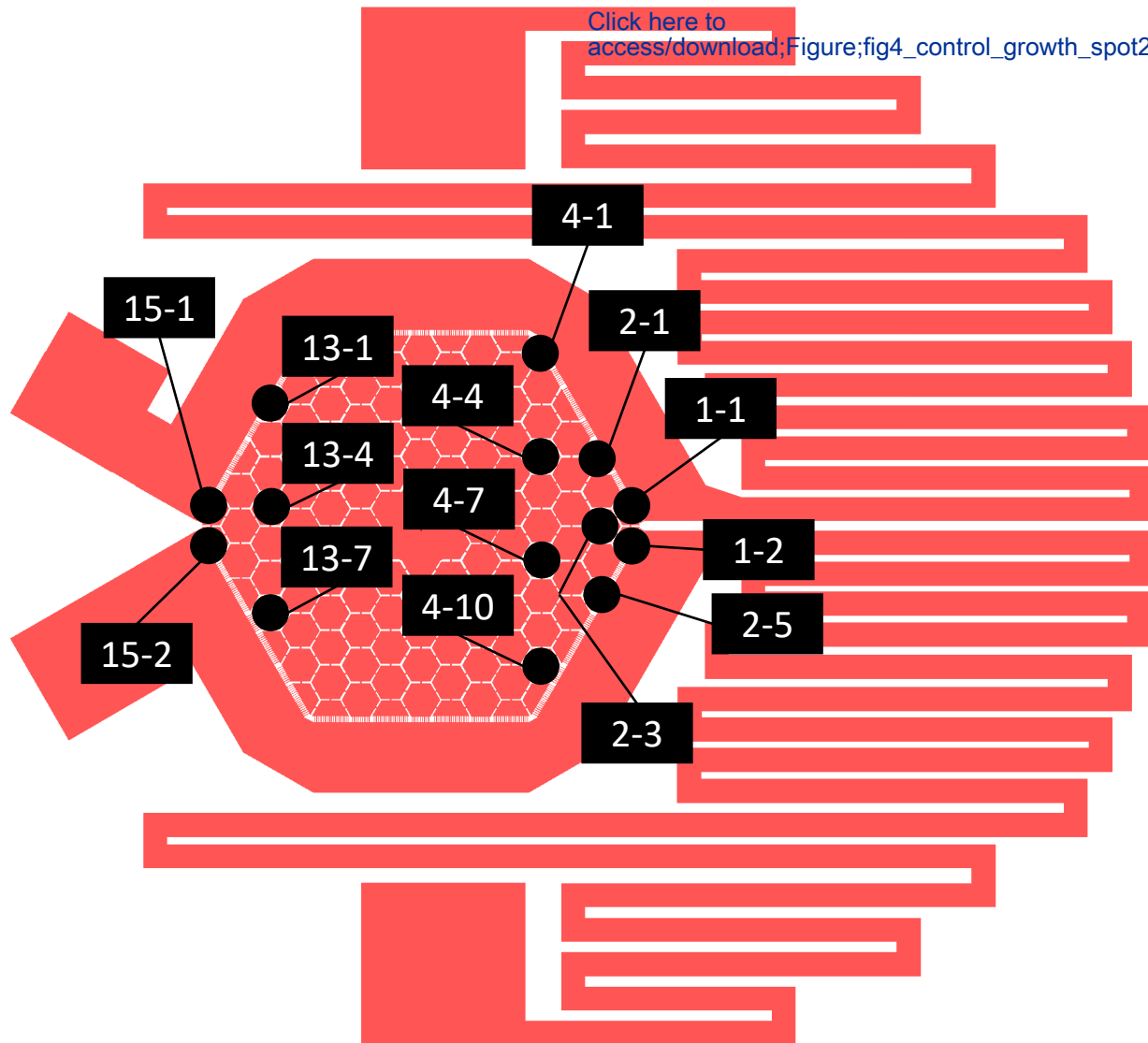


Figure 4

A



B

Cell Confluence of PC3 without Stress

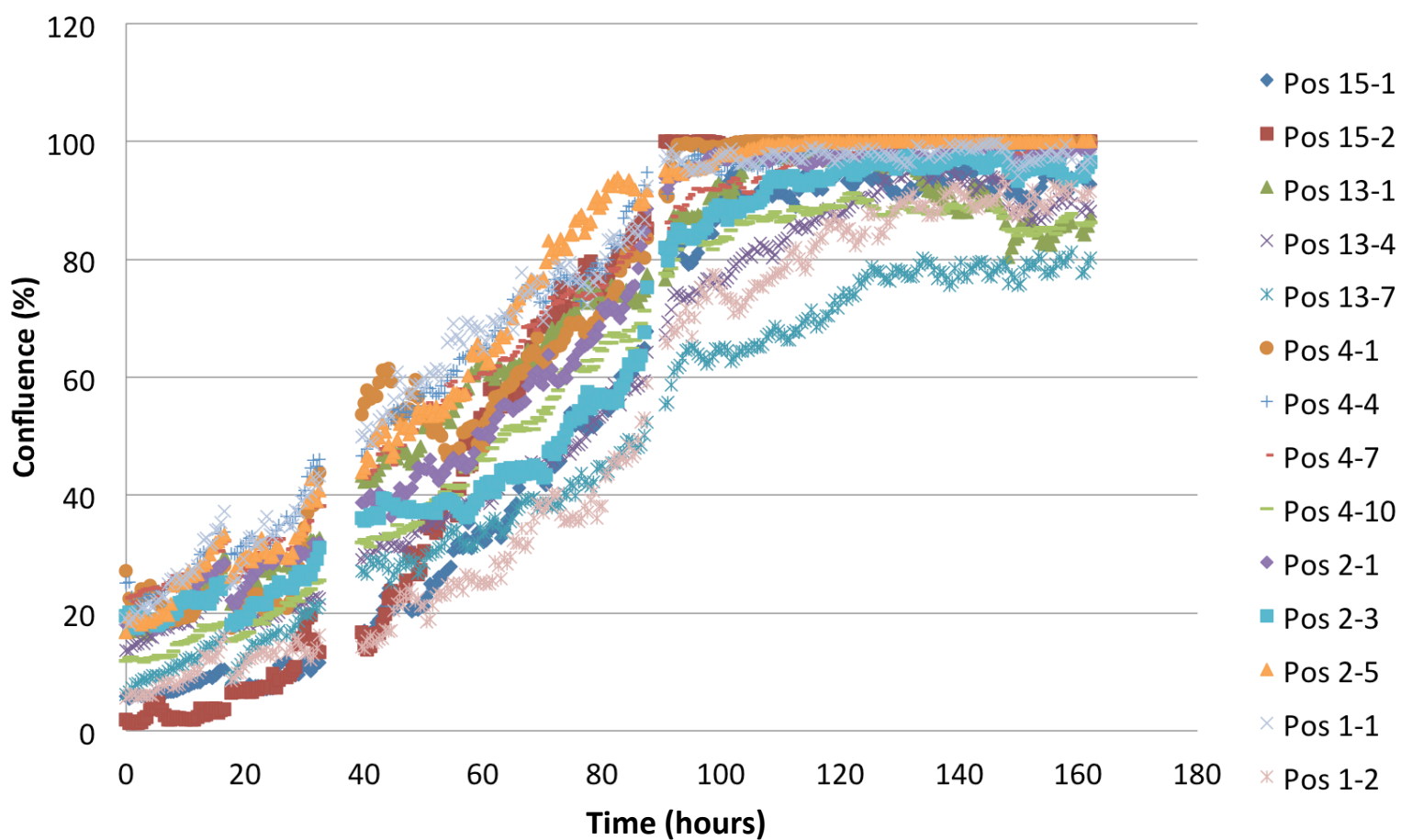
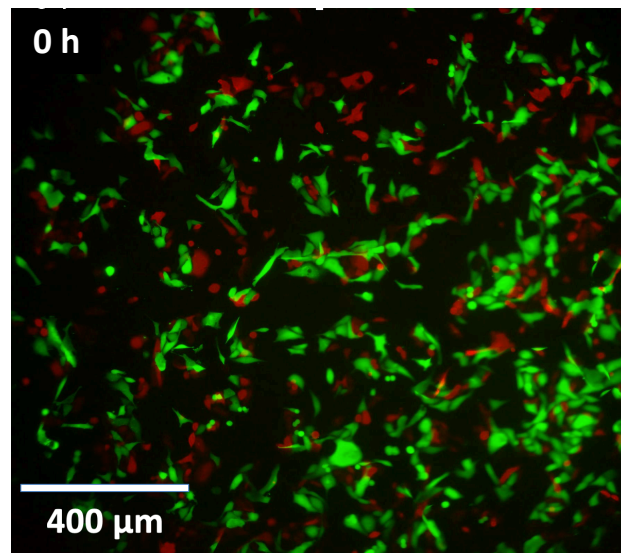
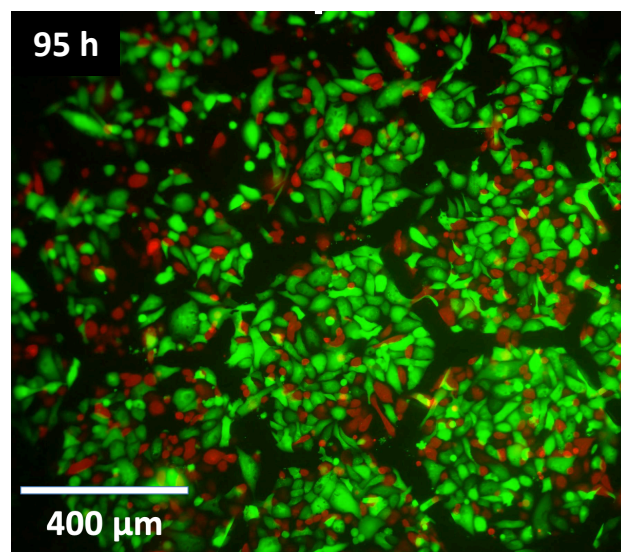


Figure 5

A



B



C

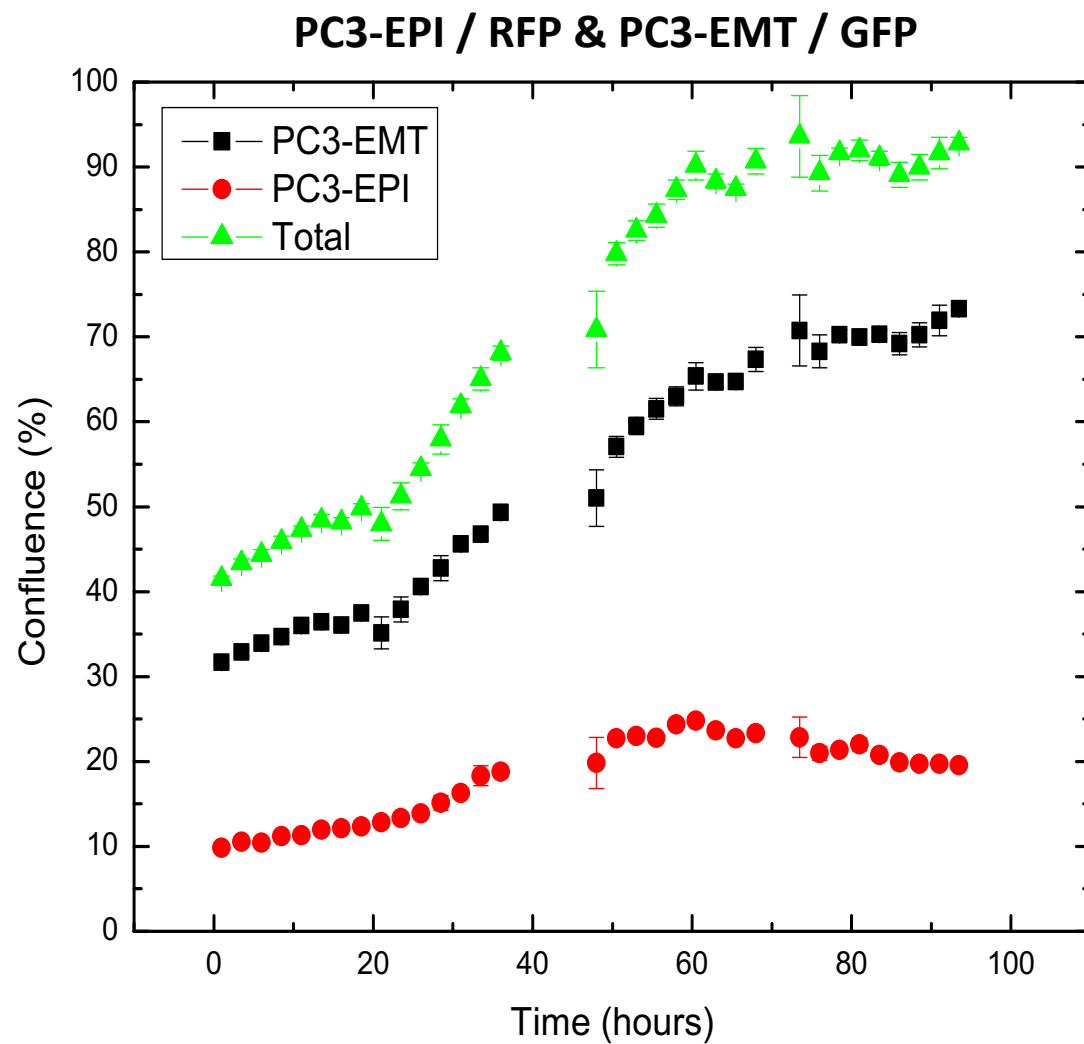
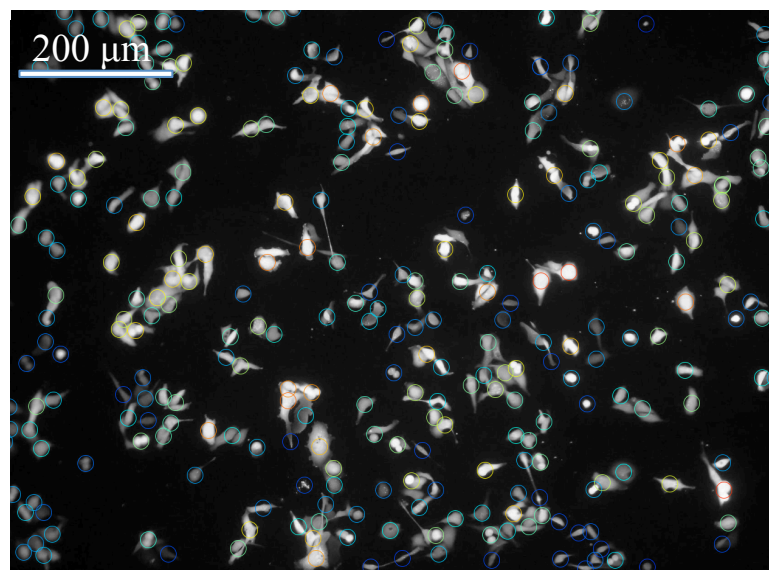
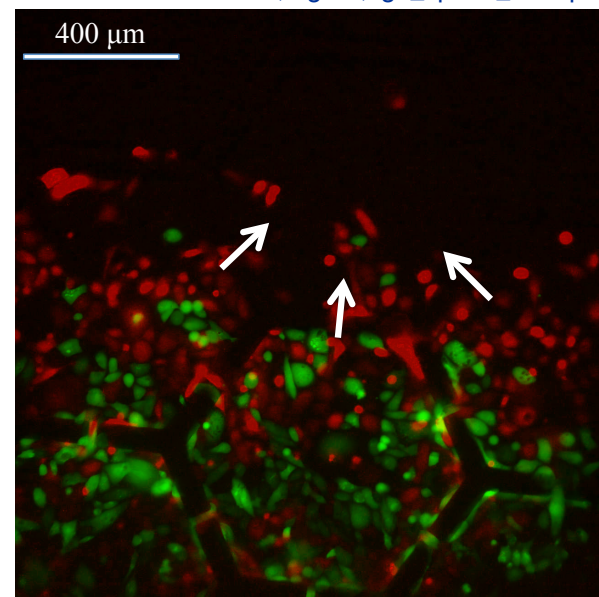


Figure 6

[Click here to access/download;Figure;fig6_speed_ver2.pdf](#)



B



C

Distribution of cellular median speed ($\mu\text{m}/\text{h}$)

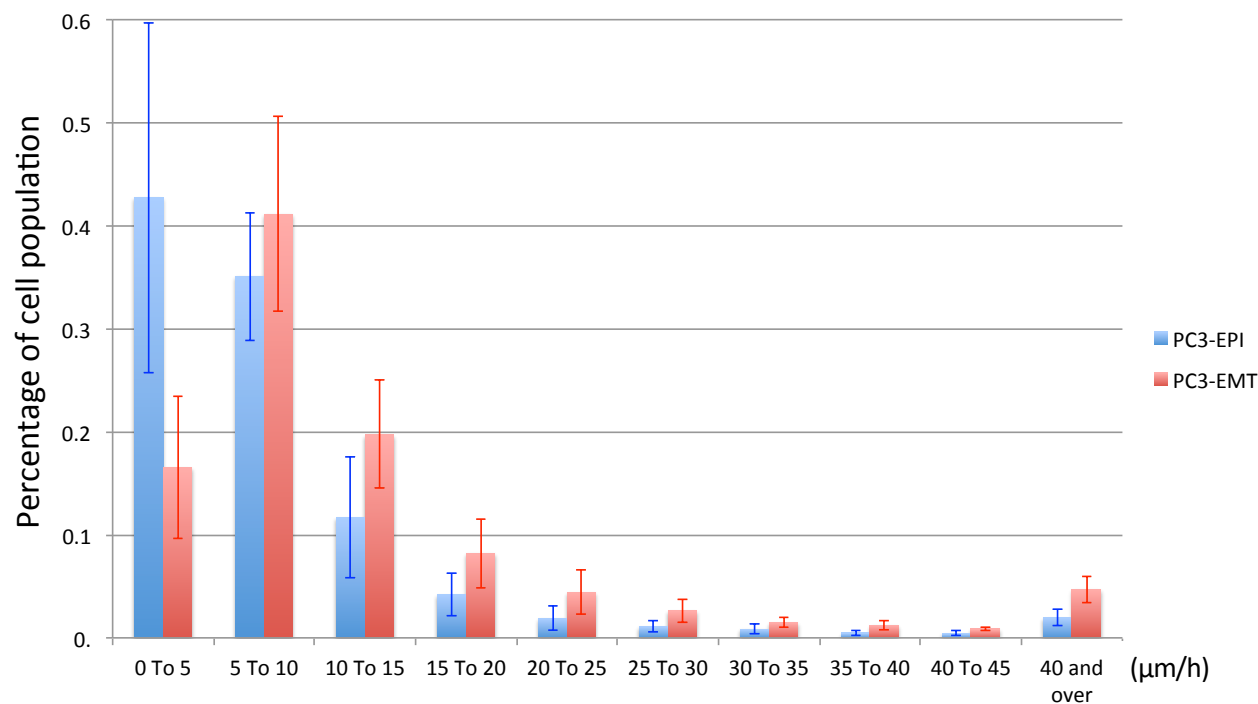
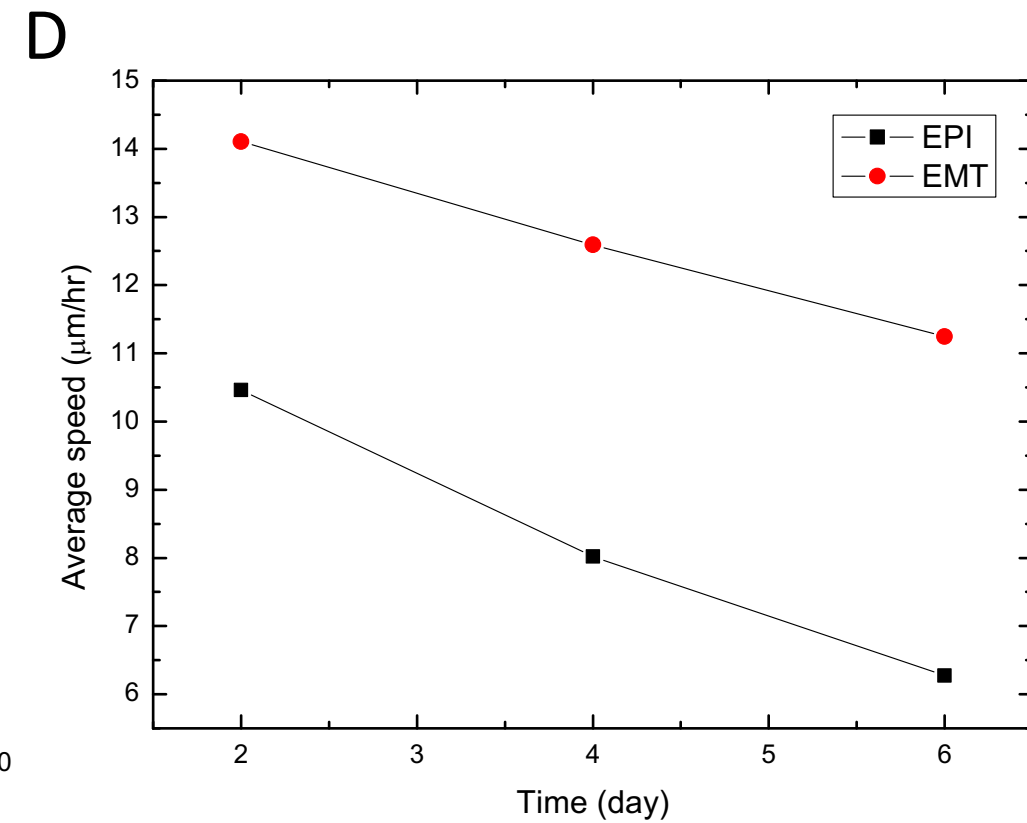
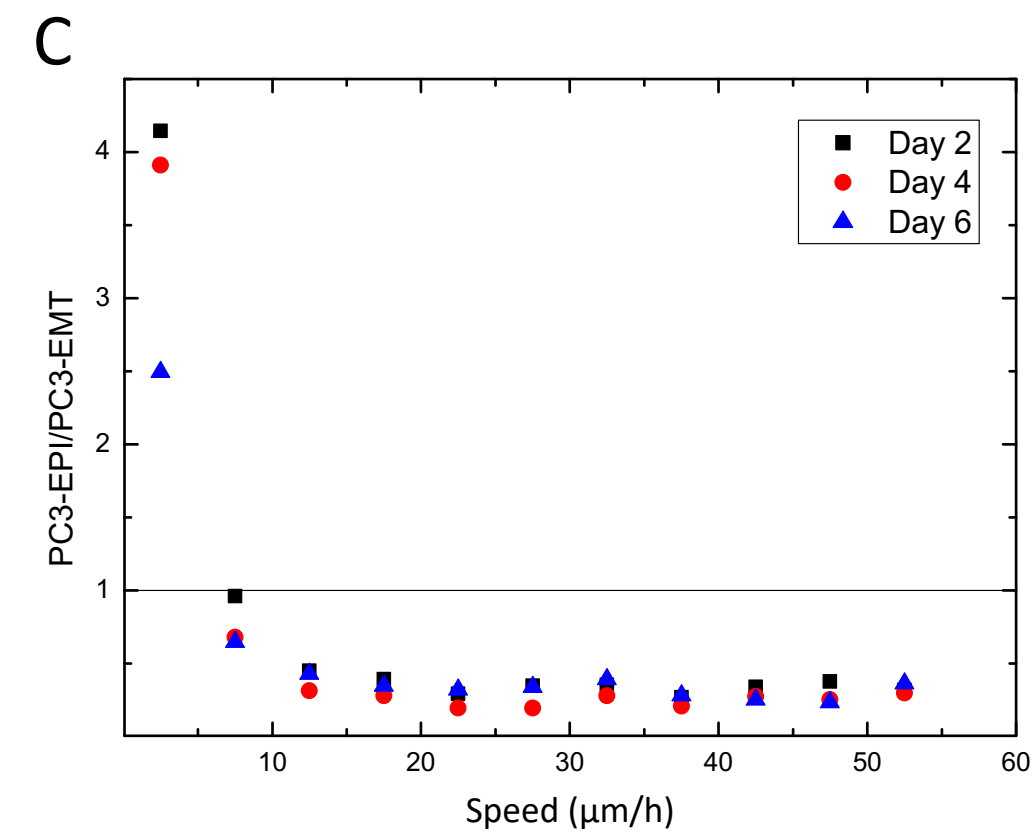
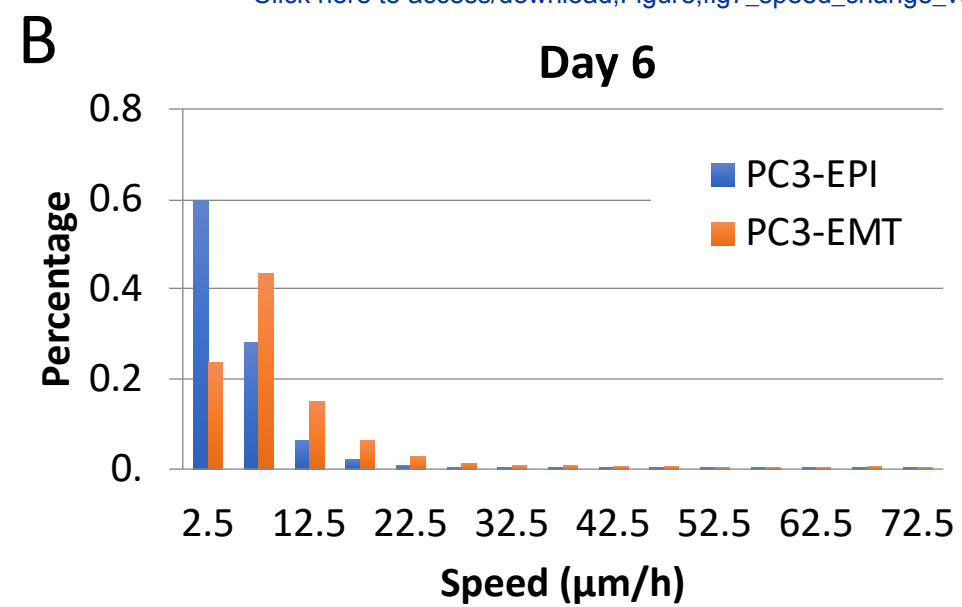
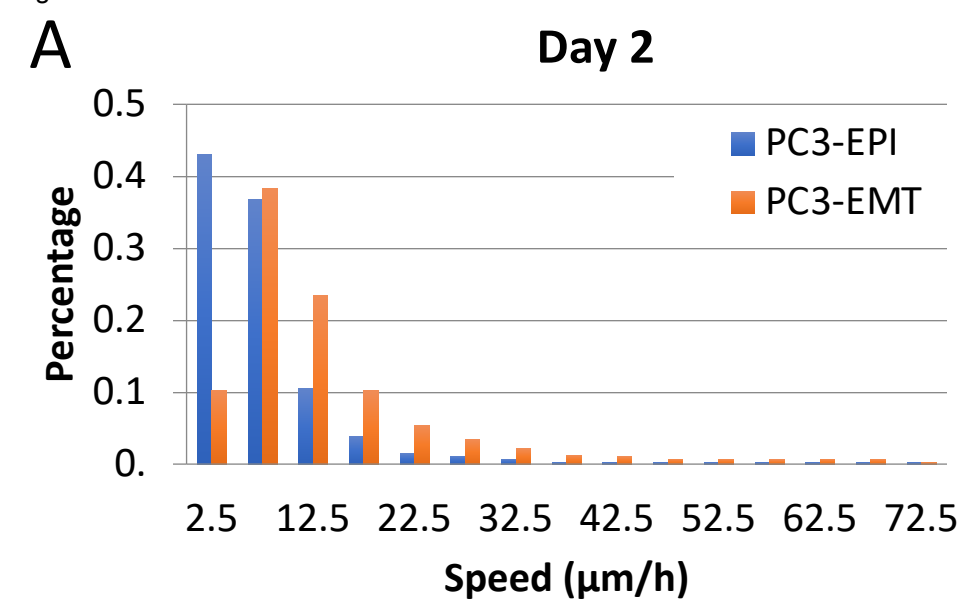


Figure 7

[Click here to access/download;Figure;fig7_speed_change_ver2.pdf](#)

Name of Material/Equipment	Company	Catalog Number
10 mL BD Luer-Lok tip syringes	BD	14-823-16E
Antibiotic-Antimycotic	Sigma-Aldrich	A5955
AZ 300 MIF	Merck KGaA	18441123163
AZ1518	Merck KGaA	AZ1518
AZ4330	Merck KGaA	AZ4330
Cr Chromium Etchant	Sigma-Aldrich	651826
Fetal bovine serum (FBS)	Life Technologies Corporation	10437028
Heidelberg DWL 66+ laserwriter	Heidelberg Instruments	DWL66+
Hexamethyldisilazane (HMDS)	Sigma-Aldrich	379212
Hollow steel pins	New England Small Tube	NE-1300-01
ibidi Heating System, Multi-Well Plates, K-Frame	ibidi	10929
Luer-Lok 23 G dispensing needle	McMaster-Carr	75165A684
Lumox dish 35	Sarstedt	94.6077.331
Microposit Remover 1165	Dow Electronic Materials	Microposit Remover 1165
Microseal B Adhesive Sealer	Bio-Rad Laboratories	MSB1001
O-Ring (for Lumox plate sealing)	McMaster-Carr	9452K114
O-Ring (for bottom glass window sealing)	McMaster-Carr	9452K74
Plasma-Preen Plasma Cleaning/Etching System	Plasmatic Systems, Inc	Plasma-Preen
RPMI 1640	Life Technologies Corporation	11875-093
Samco RIE800iPB DRIE	Samco	RIE800iPB
Suss MA6 mask aligner	SUSS MicroTec	MA6
Sylgard 184 Silicone Elastomer	Fisher Scientific	NC9285739
TePla M4L plasma etcher	PVA TePla	M4L
(PFOTS)	Sigma-Aldrich	448931
Tygon microbore tube (0.020" x 0.060"OD)	Cole-Parmer	EW-06419-01

Comments/Description
1x anti-anti
Photoresist developer
Photoresist
Photoresist
Writing photomask
For photoresist adhesion enhancement
.025 OD .017 ID x .500 long / type 304 WD fullhard
On-stage incubator
To connect syringes and tubings
Gas-permeable cell culture dish
Photoresist stripper
Adhesive sealer
Dash No. 27; 1-5/16" ID x 1-7/16" OD; Duro 70
Dash No. 20; 7/8" ID x 1" OD; Duro 70
Oxygen plasma system
Deep reactive-ion etching system
Mask aligner
PDMS elastomer
Plasma etcher
For silicon wafer silanization
Tubings for media delivery

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Signature:	Ke-Chih Lin	Date:	4/29/2019

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Manuscript No: JoVE60185

Manuscript title: Generation of heterogeneous drug stress gradients across cancer populations on the microfluidic Evolution Accelerator for real-time observation

First and corresponding author: Ke-Chih Lin

Dr. Phillip Steindel, Review Editor
Journal of Visualized Experiments

Dear Dr. Steindel,

We thank the reviewers for their thorough reading of our work and forward-looking suggestions. We have responded appropriately by adding additional information on the experimental methods and comments in the manuscript.

We have attached responses to each of the reviewers as well as the revised manuscript. Please don't hesitate to email me if I can be of further assistance.

Sincerely,
Ke-Chih Lin

Editorial comments:

General:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
2. Please ensure that the manuscript is formatted according to JoVE guidelines—letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps.
3. Please reduce the length of the summary to be between 10 and 50 words.
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(1) We have proofread the manuscript again.

(2) The manuscript is now formatted according to JoVE guidelines.

(3) The summary is now shortened (49 words).

“We present a microfluidic cancer-on-chip model, the “Evolution Accelerator” technology, which provides a controllable platform for long-term real-time quantitative studies of cancer dynamics within well-defined environmental conditions at the single-cell level. This technology is expected to

work as an in vitro model for fundamental research or pre-clinical drug development.”

(4) The format of reference numbers is now corrected.

(5)

The commercial terms have been removed and replaced with generic terms as follows:

L-Edit: removed

Heidelberg DWL66 laserwriter: laserwriter

Microposit Remover 1165: photoresist stripper

Suss MA6 mask aligner: mask aligner

Lumox™: gas-permeable culture dishes/membranes

Samco RIE800iPB DRIE: Silicon Deep Reactive Ion Etching (DRIE) systems

TePla M4L plasma etcher: plasma etcher

Polymethylsiloxane (PDMS) Sylgard 184: Polymethylsiloxane (PDMS) kit

10 mL BD Luer-Lok™ tip syringes: 10 mL syringes

Luer-Lok™ 23 G dispensing needle: 23 G dispensing needle

Microseal B Adhesive Sealer: adhesive sealer

Ibidi system: on-stage incubator

And the materials used in the manuscript have been referenced to the best of our knowledge.

Protocol:

1. For each protocol step, please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

The protocol has been revised as suggested.

Specific Protocol steps:

1. 1.1: Can you include this pattern as supplementary material?

2. 3.1.1, 3.3.1: Please include more information about the preparation of the plate holder and gas supply system.

(1) The EA pattern template files (“EA_large-gap.gds”, “EA_small-gap.gds”) and the 3D CAD file (“3-well plate.FCStd”) are now included in the supplementary materials and on GitHub.

(2) Protocol 3.1 is reorganized and supplemented. In protocol 3.3.1, our previous work is now referenced to provide more details.

Figures:

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2. Please remove the embedded table from the manuscript.

3. Please remove the embedded figure(s) from the manuscript.

4. Please consistently use either ‘h’ or ‘hours’ in the figures (not ‘hr’ or ‘hour’).

5. Figures 6c: Please define the error bars in the legend.

(1) The copyright permission of IOP Publishing obtained via Copyright Clearance Center (CCC) is uploaded as the submission material (“copyright permission.docx”).

(2) (3) The embedded table and figures are removed from the manuscript.

(4) Only 'h' and 'hours' are used in the figures now.

(5) The error bars in Fig. 6 C is now defined in the legend:

“The error bars represent the standard deviation of 5 experiment samples.”

Discussion:

1. Discussion: As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3–6 paragraphs with citations:

- a) Critical steps within the protocol
- b) Any modifications and troubleshooting of the technique
- c) Any limitations of the technique
- d) The significance with respect to existing methods

(a) Critical steps are now covered in the 3rd paragraph.

(b) Troubleshooting and modifications are now covered in the 3rd and part of the 5th paragraphs.

(c) Limitations are now covered in the 4th paragraph.

(d) The significance with respect to existing methods is covered in the 1st and the 2nd paragraphs.

References:

1. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.

(1) The format of the references is corrected.

Table of Materials:

1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

The materials used in the manuscript have been referenced to the best of our knowledge.

Manuscript No: JoVE60185

Manuscript title: Generation of heterogeneous drug stress gradients across cancer populations on the microfluidic Evolution Accelerator for real-time observation

First and corresponding author: Ke-Chih Lin

Reviewer #1:

Manuscript Summary:

The authors describe a sound protocol to prepare an evolution accelerator as a cancer cell ex vivo model.

Major Concerns:

The protocol is sound. no major concern.

Minor Concerns:

1. Practically, it will be nice if the authors can elaborate the necessity or the function for the other two stacks of PDMS(reservoir & capping layer). Was it because the tubings were not directly inserted into device? as in methods 3.4.5
2. in methods 3.4.7. For this device sealing after seeded cells approach, avoidance or clearance of microbubbles are essential because during sealing, any bubble entrapped will be significant to shear off cells or cause obstruction to the flow. Is there particular operation to avoid this. It may be critical for JOVE viewers when performing the protocol.
3. in the methods 2.2. The authors state " cell lines were transfected with cytoplasmic-labeled fluorescent marker, such that PC3-EMT express nuclear GFP and PC3-EPI express nuclear mCherry" . If the label is nuclear such as the H2B-GFP used in their paper cited as 11, then nuclear-labeled fluorescent marker will be more precise. However, after reviewing their figure 5 and 6, the fluorescent seems to be indeed cytoplasmic. It will be helpful for authors to clarify. Perhaps it's actually EF1alpha-promoted cytoplasmic fluorescent protein expression? Addition of Addgene entry of the plasmid they used for transfection will also be beneficial for interested JOVE viewer.

Authors' response: Thank you very much for your thorough reading of our work and your suggestion. We really appreciate your feedback.

(1) The description in the protocol has been modified:

“3.4.6. Prime the tubing and insert the steel pin into each PDMS chip through the capping layer. Fill up the reservoir layer and wet the PDMS pattern layer pattern with media. The reservoir layer works as an on-chip bubble trap to prevent air bubbles from getting into the microfluidic pattern.”

(2) The description in the protocol has been modified:

“3.4.10. Place chip directly on top of the gas-permeable culture membranes (with cells already adhered to the membranes). In order to avoid entrapping microbubbles in the microfluidic pattern, dispense 1 mL prewarmed and degassed media into the 35-mm gas-permeable culture dish before assembly, and then make sure that the chip approaches the liquid surface with a 15-degree tilt angle.”

(3) Thank you for pointing it out. This is actually a typo. There is no H2B label used in this paper. The description in the protocol has been modified as follows:

“2.2. Cell lines were transfected with cytoplasmic-labeled fluorescent markers for better visualization, as described previously¹², such that PC3-EMT expresses cytoplasmic GFP and PC3-EPI expresses cytoplasmic mCherry. Note that the technology is also compatible with any fluorescent-labeled cells as well as brightfield imaging of unlabeled cells.”

Manuscript No: JoVE60185

Manuscript title: Generation of heterogeneous drug stress gradients across cancer populations on the microfluidic Evolution Accelerator for real-time observation

First and corresponding author: Ke-Chih Lin

Reviewer #2:

Major Concerns:

None.

Minor Concerns:

This paper introduces the Evolutionary Accelerator, a microfluidic cancer-on-chip model which reproduces important features of a complex tumor microenvironment, while enabling robust quantitative descriptions of cancer dynamics. Specifically, it allows cancer cells to inhabit an interconnected array of microenvironments while generating a heterogeneous concentration landscape (of chemotherapeutics and other chemical stress, or possibly nutrients). This allows long-term, real-time monitoring of cancer evolutionary dynamics.

This is an innovative and elegant system that is utterly needed considering the high cost and difficulties of mouse models in addition to their questionable relevance to human cancer progression on the one hand, and the insufficient complexity of 2D cultures on the other hand. The system and its capabilities are very well presented through images, the protocol and demonstrations (cell growth and motility assays). Therefore, I recommend publication, while suggesting that the Authors address the following comments (optional).

(1) Line 293: doubling time in conventional cultureware is 24 hours. Can this be supported by data or a reference?

(2) A review paper that may be relevant and may be worth citing is, "Towards control of cellular decision-making networks in the epithelial-to-mesenchymal transition", *Phys Biol.* 16(3):031002 (2019).

(3) "Microfluidic cancer-on-chip models 38 have been prophesied to bridge the gap between the oversimplified conventional 2D cultures" -prophesied should probably be proposed.

(4) The system is an "evolution accelerator" - a few words on how it accelerates evolution, referring to earlier work (Death Galaxy) by the authors would be great.

(5) Are the microfluidic pattern template file and the plate holder pattern available?

(6) More information would be useful on point 3.2.5 in disinfecting with UV exposure- for how long?

(7) May be worth mentioning that not all 3 lumox dishes/chambers need to be used at the same time.

(8) It would be useful to clarify if any parts of the protocol are optional, and which are interchangeable. Are any variables interchangeable or modifiable? E.g., 3.4.10 flow rate, does it have to be 20uL/hr?

(9) Microscopes reliant on perfect focus, won't be able to focus on the interface of the PDMS/lumox chip automatically. The authors should cover this information in detail and suggest solutions. In general, they should comment on the portability of the system to a standard research lab.

(10) Are there any regions in the chip where cells did not grow well?

(11) Do the authors observe wash off of cells that escape out of the culture region? If so, how common is it and how much of the population can be lost in such a way?

Authors' response: Thank you very much for your kind words and comments. We really appreciate your feedback.

(1) A paper has been referred to support the presented doubling time:

¹³ Lin, K. C. et al. The role of heterogeneous environment and docetaxel gradient in the emergence of polyploid, mesenchymal and resistant prostate cancer cells. *Clinical & experimental metastasis*. **36** (2), 97-108 (2019).

(2) The suggested review article is now cited in the Discussion section:

“With the ability to control and monitor the behaviors of mixed tumor cell populations under well-controlled stress gradients, our microfluidic EA technology may also serve as a platform to study regulatory mechanisms and the phenotypic transformation involved in EMT in the context of cancer therapeutic strategies.²²”

(3) The sentence now reads: Microfluidic cancer-on-chip models have been proposed to bridge the gap ...

(4) A few sentences have been added in the second paragraph of the Introduction section:

“The design of the Evolution Accelerator is based on our previous work, in which the evolutionary dynamics of organisms in a metapopulation can be accelerated.¹⁰⁻¹¹ Specifically, in a group of spatially separated populations which interact at some level, when exposed to a heterogeneous stress landscape, the most fit species can dominate in a local population faster compared with that of a large uniform population. The advantageous species then migrate to neighboring microhabitats in search of resources and space, and eventually dominate the entire population.”

(5) The EA pattern template files (“EA_large-gap.gds”, “EA_small-gap.gds”) and the 3D CAD file (“3-well plate.FCStd”) are now included in the supplementary materials and on GitHub.

(6) The description in the protocol has been modified:

“3.2.5. Disinfect the PDMS devices via UV exposure for at least 1 hour and leave in a sterile environment.”

(7) A sentence has been added in the protocol:

“3.4.2. ... Note that the 3 wells are independent, and 3 experiments can be done separately.”

(8) The variables and procedures in the protocols have been optimized. We suggest the readers to follow our recipe if there's no special requirement. For instance, if the flow rate is set to be higher than 100 $\mu\text{L/hr}$, the cells near the peripheral habitats would be affected by the advection flow and do not grow well. If the flow rate is set to be lower than 1 $\mu\text{L/hr}$, the flow is not sufficient to maintain fixed bio-chemical boundary condition in the media channels.

(9) More description is now added in the protocol:

4.2. Configure software to acquire images across two channels at 10X magnification for each chip with automatic image-stitching after one round of autofocus. Be aware that Perfect Focus does not guarantee satisfying image quality. It is more recommended to generate a customized focus surface for long-term image acquisition based on either autofocus or manual focus across the chip.

4.3. Take images every hour and leave experiment running on the time scale of weeks. Monitor image quality on a daily basis and update the focus surface if necessary.


(10) If strictly following the suggested protocols, there should be no obvious variation in terms of cell proliferation rate across the chip.

(11) The width of the slits (15 μm) is design to prevent cells from escaping the culture region, but this can definitely happen. The portion of the cells that “extravasated” into the media channels depends heavily on cell types. Typically, the probability of each cell getting into the media channels ranges from 0.005 to 0.02 (1/hour). Mesenchymal cells have higher escaping rate than epithelial phenotypes.



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