Supplementary Materials I

Design and Fabrication of 3D Microfluidic Cell Arrays

Masks of three layers of a 3D μFCA were designed in AutoCAD. Glass masks with Cr coating were manufactured at Cornell Nanoscale Facility. Silicon etching was employed in master making. Scanning electron microscopy (SEM) was used to characterize surface features on each master. The thickness of features on masters was measured using a profilometer (Dektak 150, Veeco, Plainview NY). They are 100 μm for microchambers, 60 μm for pillars on the middle filter layer master, and 130 μm for microchannels.

To generate the bottom microchamber layer and middle filter membrane, the corresponding masters were spin coated with PDMS to obtain desired thicknesses, such as 80 μm for the bottom microchamber layer and 40 μm for the middle filter membrane. The PDMS layers were cured for 4 hours at 80 ^{o}C in an oven. The top PDMS microchannel layer was cut and removed from its master. The inlet and outlet holes for medium flow were perforated using 21 gauge needles (Small Parts Inc., Logansport, IN). Then it was bonded on top of the middle PDMS filter membrane. Oxygen plasma treatment was used before each bonding. The bonded top and middle PDMS layers were removed from the middle layer master, and bonded on the bottom PDMS microchamber layer. Alignments among microchannels, pores and microchambers in three layers were performed under a stereomicroscope. The stack of three layers was removed from the silicon master of the bottom layer. Inlet-outlet holes for cell seeding were perforated. Then this three-layer PDMS device was bonded to a microscope cover glass (VWR, Bridgeport NJ).

Cell Culture

Three types of cells were used in this study, human ductal breast epithelial tumor cell line (T47D), human non-small cell lung cancer cell line (PC9), and adult human dermal blood

microvascular endothelial cells (HMVEC) (Lonza, Allendale NJ). The cell culture medium of T47D is 50% DMEM (Invitrogen, Grand Island NY) and 50% F12K (ATCC, Manassas VA), RPMI-1640 (ATCC) for PC9, and HMVEC medium (Lonza) supplied with growth factors (Lonza) for HMVEC- dBlAd. All media were supplied with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA), and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA). All cells were cultured in an incubator at 37 ^{o}C with 5% CO_2.

Cell Seeding in 3D μFCA

A 3D μFCA was sterilized with UV overnight in a cell culture hood and rinsed with sterilized water. The whole device was first filled with water for degassing. Then inlets and outlets of the top layer were clamped to prevent pressure gradients between two layers. Cancer cells encapsulated in 0.25% PuraMatrix (BD Biosciences, Franklin Lakes NJ) were introduced to microchambers using tubing through inlets/outlets of the bottom layer. Since there is no pressure gradient between the top and bottom layers, the liquid form of the cell and PuraMatrix mixturein the bottom layer does not flow through pores to the top. PuraMatrix is a synthetic peptide hydrogel with four repeats of amino acids RAD. It was purchased as 1% in water and its gelation can be induced by physiological salt solutions[1] to form 3D interweaving nanofiber scaffold[2]. The fiber diameter and pore sizes in the hydrogel are about 10 nm and 50-200 nm respectively. PuraMatrix promotes cell attachment, migration and retain cellular phenotypes across a number of cell types, such as endothelial cells [3], hepatocytes[4], chondrocytes[5], osteoblasts[6], mesenchymal stem cells[7] and cancer cells[8, 9]. Its storage modulus at 0.5% is 2.5kPa[3]. Other types of hydrogel (e.g. collagen) can also be used to encapsulate and culture cells in our 3D μFCA. PuraMatrix was chosen as 3D cell encapsulation matrix due to its properties of extremely low autofluorescence and insensitive to temperature during gelation. After cell seeding, the inlets/outlets of the bottom

layer were clamped. The clamping of inlets and outlets of the top layer was released, then cell culture medium was introduced to microchannels in the top layer via tubing connected to a syringe pump with 0.5 µl/min flow rate. The continuous medium flow triggers PuraMatrix gelation and maintains cell culture by diffusion between top and bottom layers. The calculated shear stress (0.07 dyn/cm^2) was lower than the range of physiological values of microvessels and capillaries[10]. We prefer the low shear stress to maintain endothelial cell adherence to the top of the filter membrane. The entire system comprising of the cell-seeded 3D µFCA and pump was maintained in a regular cell culture incubator.

Viability of Cancer Cell Culture in 3D µFCA

T47D cells encapsulated in 0.25% PuraMatrix at 10 million/ml were cultured for 7 days in a 3D µFCA device. On Day 7, 4 µM Calcein AM (Invitrogen, Grand Island NY) in medium was introduced to microchannels in the top layer to show the cell viability and further confirm the vertical diffusion between top and bottom layers. Live cells become fluorescence green upon uptaking calcein AM. Time-lapse images of green fluorescence in the first 15 minutes after the Calcein AM introduction were captured with one second interval to monitor cells in 3D µFCA in real time using a Zeiss Observer Z.1 (Carl Zeiss, Germany).

Long Term Cell Culturing in 3D µFCA

Long term viability of cells in a 3D µFCA is critical for accurate drug screenings and measurements of tumor pathology. PC9 cells at 60 million/ml were stained with DiI fluorescence cell tracker (Invitrogen; Grand Island NY), encapsulated in 0.25 % PuraMatrix, and seeded in a 3D µFCA. PC9 cells were cultured in the 3D µFCA for 13 days at 37 ^oC in 5% CO_2. Cells were fed through a continuous perfusion of fresh medium at 0.5 µl/min in the top microchannels. PC9 cells were observed at Day1, Day 7 and Day 13 using a Zeiss Observer Z.1.

On Day 13, 4 μM Calcein AM was introduced to microchannels in the top layer to show the long term cell viability. 3D DiI images on Day 1, 7, and 13 from three microchambers were analyzed to estimate the PC9 cell growth rate in a 3D μFCA. Both short and long term cell viability tests in our devices were repeated more than 3 times. 3D μFCAs were disposed after each experiment.

Structured Coculture in 3D μFCA

3D μFCA is specifically designed with the impact of microenvironment on tumor cells in mind. This is achieved by co-culturing different types of cells within the fabricated multiple layers. PC9 cells stained with DiI and embedded in 0.25 % Puramatrix were seeded in the microchambers of 3D μFCA following the same procedure as before. The next day HMVECs were seeded inside the microchannels in the top layer, after they were coated with 30 μg/ml fibronectin (Sigma, St Louis MO). The two types of cells were cocultured in a 3D μFCA at 37 ^{o}C in 5% CO_2 for 3 days under continuous medium flow in microchannels before drug testing. The ratio of seeding densities between HMVECs on the top layer and PC9 inside the Puramatrix gel was ~40%, which is within the ratio range of stromal cells to tumor cells (~20% - 55%)[11]. Phase contrast and fluorescence Z-stack images of PC9 cells and HMVECs were taken under Zeiss Observer Z.1.

Caspase-3 activity measurement in different cell cultures

Four apoptotic inducers were applied to compare the caspase-3 activities of PC9 cell cultures in conventional culture dishes with that in the 3D tumor microenvironment generated in a 3D μFCA. Tarceva® (200nM) (Genentech Inc., South San Francisco CA), staurosporine (1μM) (Enzo, Farmingdale NY), TNF-α (Tumor Necrosis Factor) (15ng/ml) (R&D Systems, Minneapolis MN) with cycloheximide (2μg/ml) (Sigma, St Louis MO), and colchicine (0.6μM) (BIOMOL, Farmingdale NY) were used. Caspase-3 activities were monitored as an apoptotic

marker because of its critical role for ensuring completion of the apoptotic process[12]. Caspase-3 activities were measured using DEVD-Nucview 488 (Biotium, Inc., Hayward CA). The kit was first evaluated in PC9 cells of the conventional monolayer (2D) culture with the same four drugs plus two control conditions (i.e. no drug and no drug + caspase-3 inhibitor) using flow cytometry. Eight hours after drug treatments, PC9 cells of 2D cultures with DEVD-Nucview 488 were analyzed by a flow cytometer, FACSCalibur (Becton Dickinson, Franklin Lakes NJ). FACS analysis suggested that PC9 cells had different temporal caspase-3 activation profiles in response to these four drug perturbations in the 2D monolayer cultures.

24-well tissue culture plates were used for conventional cell cultures, which included 90% confluent monolayer (2D) culture, 3D PC9 encapsulation in 0.25% PuraMatrix and 3D coculture of PC9 and HMVECs. In the case of conventional 3D coculture of PC9& HMVECs, HMVECs were seeded on top of the PC9 cells embedded in PuraMatrix one day after the PC9 encapsulation, the exact same cell seeding order as that of the structured coculture in a 3D μFCA.

Image Capture for 3D Cell Culture

A Zeiss epi-fluorescence microscope (Observer Z.1) equipped with a function of an objective moving in the z direction and Axio Vision 4.7 (Carl Zeiss, Germany) software were used to take wide-field z-stack images for 3D cell culture. The z-slicing function on an epi-fluorescence microscope uses the similar theory as confocal for z-direction scanning but without the second focal point before an objective. Therefore, deconvolution software has to be used to generate clear 3D cell/tumor images from z-stack images suitable for visualizing cells in their 3D matrix. For experiments of caspase 3 activities, time-lapse images up to 14 to17 hours with one hour intervals during the drug treatment and z-stack images were also captured at multiple

locations. An on-stage incubator was used to keep cells at 37 °C with humidified 5% CO_2 during the dynamic drug stimulation experiments.

Image Analysis and Quantification

For 3D z-stack images deconvolution was performed using Axiovision 4.7 with inverse filter image restoration algorithm for rapid contrast improvement and image sharpening. Fluorescence images were quantified using custom image analysis routines written in MATLAB (Mathworks Inc., Natick MA) for color conversion and background elimination. Then CTan (Skyscan Inc., Kontich Belgium) was used to obtain fluorescence intensity measurement. 2D Images were acquired from four individual positions in each well. 3D images for the 3D tissue culture plate samples were acquired in two individual positions from each well of two wells, for each drug per time. From the 3D μFCA device experiments, 3D images of four microchambers for each drug per time point were analyzed. Data obtained from such different positions were used to calculate the average fluorescence intensity per time point. Each response data was normalized with nondrug control series to facilitate comparison between the groups with different culture conditions. The normalized data expressed as relative caspase-3 activities were logarithmic results of ratios of fluorescence intensity of treated samples to no-drug controls respect to base 2. Since the cell seeding in our device was through viscous cell-gel mixture, there was no visible cell density variation among the different microchambers. It is reasonable to assume that there are the same number of cells in each well and that there are enough cells per well to represent the average response on a 3D μFCA in each experiment. Normalizing makes responses independent of the number of cells in each group.

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