

A 3D biophysical model for cancer spheroid cell-enhanced invasion in collagen-oriented fiber microenvironment

Miaomiao Hai(海苗苗), Yanping Liu(刘艳平), Ling Xiong(熊玲), Guoqiang Li(李国强), Gao Wang(王高), Hongfei Zhang(张鸿飞), Jianwei Shuai(帅建伟), Guo Chen(陈果), Liyu Liu(刘雳宇)

Citation: Chin. Phys. B . 2020, 29(9): 098702 . **doi:** 10.1088/1674-1056/ab9c02
Journal homepage: http://cpb.iphy.ac.cn; http://iopscience.iop.org/cpb

What follows is a list of articles you may be interested in

Controlled generation of cell-laden hydrogel microspheres with core-shell scaffold mimicking microenvironment of tumor

Yuenan Li(李岳南), Miaomiao Hai(海苗苗), Yu Zhao(赵宇), Yalei Lv(吕亚蕾), Yi He(何益), Guo Chen(陈果), Liyu Liu(刘雳宇), Ruchuan Liu(刘如川), Guigen Zhang

Chin. Phys. B . 2018, 27(12): 128703 . **doi:** 10.1088/1674-1056/27/12/128703

Amyloid-*β* peptide aggregation and the influence of carbon nanoparticles

Wen-Hui Xi(郗文辉) and Guang-Hong Wei(韦广红)

Chin. Phys. B . 2016, 25(1): 018704 . **doi:** 10.1088/1674-1056/25/1/018704

Hierarchical processes in *β* -sheet peptide self-assembly from the microscopic to the mesoscopic level

Li Deng(邓礼) and Hai Xu(徐海)

Chin. Phys. B . 2016, 25(1): 018701 . **doi:** 10.1088/1674-1056/25/1/018701

A 3D biophysical model for cancer spheroid cell-enhanced invasion in collagen-oriented fiber microenvironment*

Miaomiao Hai(海苗苗)[1,†], Yanping Liu(刘艳平)[1,†], Ling Xiong(熊玲)[1], Guoqiang Li(李国强)[1], Gao Wang(王高)[1], Hongfei Zhang(张鸿飞)[2], Jianwei Shuai(帅建伟)[3], Guo Chen(陈果)[1], and Liyu Liu(刘雳宇)[1,‡]

[1] *Chongqing Key Laboratory of Soft Condensed Matter Physics and Smart Materials, College of Physics, Chongqing University, Chongqing* 401331, *China*
[2] *Hygeia International Cancer Hospital, Chongqing* 401331, *China*
[3] *Department of Physics, Xiamen University, Xiamen* 361005, *China*

(Received 29 April 2020; revised manuscript received 22 May 2020; accepted manuscript online 12 June 2020)

The process of *in situ* tumors developing into malignant tumors and exhibiting invasive behavior is extremely complicated . From a biophysical point of view, it is a phase change process affected by many factors, including cell-to-cell, cell-to-chemical material, cell-to-environment interaction, *etc*. In this study, we constructed spheroids based on green fluorescence metastatic breast cancer cells MDA-MB-231 to simulate malignant tumors *in vitro*, while constructed a three-dimensional (3D) biochip to simulate a micro-environment for the growth and invasion of spheroids. In the experiment, the 3D spheroid was implanted into the chip, and the oriented collagen fibers controlled by collagen concentration and injection rate could guide the MDA-MB-231 cells in the spheroid to undergo directional invasion. The experiment showed that the oriented fibers greatly accelerated the invasion speed of MDA-MB-231 cells compared with the traditional uniform tumor micro-environment, namely obvious invasive branches appeared on the spheroids within 24 hours. In order to analyze this interesting phenomenon, we have developed a quantitative analyzing approach to explore strong angle correlation between the orientation of collagen fibers and invasive direction of cancer cell. The results showed that the oriented collagen fibers produced by the chip can greatly stimulate the invasion potential of cancer cells. This biochip is not only conducive to modeling cancer cell metastasis and studying cell invasion mechanisms, but also has the potential to build a quantitative evaluation platform that can be used in future chemical drug treatments.

Keywords: 3D biochip, spheroids, MDA-MB-231 cells, oriented collagen fibers, cancer cell invasion

PACS: 87.18.Gh, 87.14.em **DOI:** 10.1088/1674-1056/ab9c02

1. Introduction

Cancer has become a major health problem worldwide with increasing morbidity and mortality, and has attracted widespread attentions. Breast cancer is one of the most common cancers, which accounts for 30% of the confirmed cancer cases in 2019.[1] The main threat of cancer is the high fatality rate, especially as cancer cells invade and metastasize, causing up to 90% of death of cancer patients.[2,3] In clinic, tumor growth in primary sites could be surgically removed at its early stage.[4] However, after the tumor has developed to a certain extent, the cancer cells will detach from the primary tumor site and transfer to the extracellular matrix (ECM), which leads to the surgically removing invalid.

Figure 1 is a schematic diagram of tumor local invasion and infiltration *in vivo*. At the first stage, the tumor is surrounded by a rich extracellular matrix, and these collagen fibers are randomly oriented (black). At the second stage, the cancer cells start to invade locally and change the orientation of the collagen fibers (green fibers) with the growth of the tumor. Meanwhile, the presence of the nutritional gradient would establish rapidly along the collagen fibers. At the third stage, cancer cells destroy the blood vessel wall, penetrate the blood vessel and migrate to other parts of the body with the circulation of blood or lymph fluid. The result is to damage other parts and functions of the body, leading to the death of the patient. Due to the randomly scattered sites of cancer metastasis, it is difficult to achieve the desired results with single surgery, chemotherapy, drugs, *etc*.[5] Therefore, it is essential to develop clinically more effective treatment methods based on the characteristics of tumor and invasion micro-environment.

Culturing single or multiple cancer cells is the most commonly used method for *in vitro* studies of the cancer invasion. Cells will attach to the bottom of the culture dish in the traditional two-dimensional (2D) cell culture system, which renders the 2D cell culture many limitations, such as the lack of cell–cell and cell–ECM interactions. What is more, the invasion, metastasis, and the effects of drugs may be restricted.[6]

*Project supported by the National Natural Science Foundation of China (Grant Nos. 11974066 and 11674043), the Fundamental Research Funds for the Central Universities, China (Grant No. 2019CDYGYB007), and the Natural Science Foundation of Chongqing, China (Grant No. cstc2019jcyj-msxmX0477).

†These authors contributed equally to this work.
‡Corresponding author. E-mail: lyliu@cqu.edu.cn

© 2020 Chinese Physical Society and IOP Publishing Ltd

Here, a three-dimensional (3D) spheroid can better simulate tumors, which is increasingly recognized as an effective way to study the complex growth and development of tumors,[7–9] and the most advanced anti-cancer drug test and screening platform.[10,11] It fills the gap of traditional 2D *in vitro* culture and animal models.[12] Through 3D cell culture, the morphology, function, and characteristics of tumor tissue *in vivo* can be simulated *in vitro*, such as cell–cell interaction, cell migration, cell signaling, drug penetration, response, and drug resistance.[13,14]

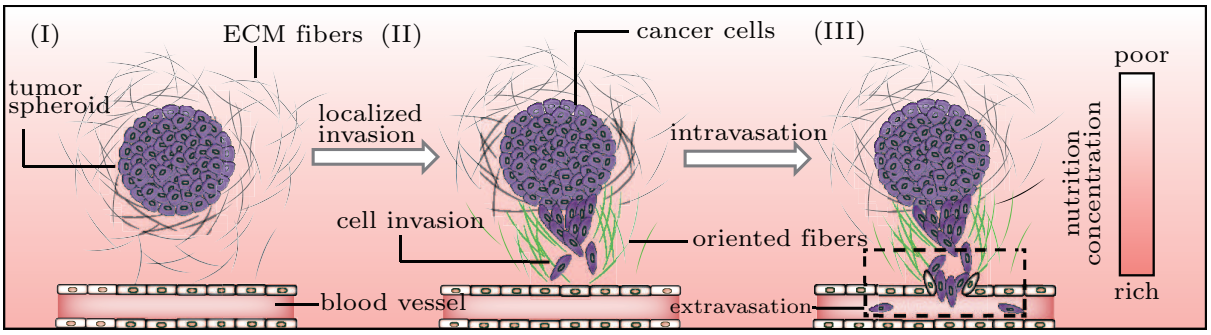


Fig. 1. The multiple stages of metastatic dissemination of spheroid from the primary tumor into the blood circulation. (i) Non-invasive tumor spheroid surrounded by collagen fibers (black). (II) Local invasion occurs on the cancer spheroid, and the collagen fibers surrounding the branches have orientation. (III) The cells at the front of the invading branch of the cancer spheroid invade the nutrient-rich blood vessels. Metastatic cancer cells rapidly invade by changing the orientation of collagen fibers.

At present, commonly used methods for generating tumor spheroids include: hanging drop, rotating flask, rotating cell culture system, ultra-low attachment plate, and microfluidic. Each method has its advantages and limitations. The hanging drop method is relatively simple and suitable for many cell lines. The resulting spheroid is tightly packed, and they show strong stability in size. The volume of cells containing droplets is generally 15 μL–30 μL (including drug test media), because the surface tension of the liquid that remains attached to the culture surface does not support a larger volume.[15] The spinner bottle bioreactor works in a similar manner. The advantage is that it can produce a large number of spheroids, and the medium can be changed frequently to support the long-term cultivation of 3D cancer cell spheroids, *etc*.[16] The attachment plate method is simple and reproducible, because it is possible to strictly control the inoculation of the same number of cells in each well of the well plate, which can produce cancer cell spheroids of uniform size and shape.[17] The advantage of microfluidic method is to repeat multiple experiments on a chip to ensure the accuracy of experimental data. The technique does not require special equipment, and the cells can be easily observed under the microscope, and the required liquid sample is very small. The disadvantage is that because the sample volume generated by each plate is small, and the further analysis of samples (such as immunoblotting) based on the microfluidic chip may be difficult.[18] Therefore, there is a lack of standard and rapid methods for laboratory research to simply control the size and shape of cancer cell spheroids within a short preparation time.

At the same time, the micro-environment must be considered when studying the invasion of cancer cells *in vitro*. The microenvironment of *in vivo* invasion is more complicated, and the extracellular matrix is an important part of the microenvironment. The occurrence, development, invasion, and metastasis of malignant tumors are often accompanied by changes in the extracellular matrix.[19,20] The extracellular matrix is mainly composed of four types of substances, namely collagen, elastin, proteoglycan, and aminoglycan, which are the basement membrane at the base of epithelial or endothelial cells, and the intercellular adhesion structure in the interstitial connective tissue.[21,22]

According to recent studies, it has been confirmed that specific proteins on invasive cancer cells can induce cancer cell invasion by binding collagen fibers, thereby changing the direction of collagen fibers.[23] Many studies on the impact of cancer cell complex microenvironment on the invasion of cancer cells are based on organ-on-chip with specific functions. These chips have more controllability, quantification, and better control ability than 2D cell culture dishes, which can build a more complex cell microenvironment, such as lung on a chip reconstructing the organ-level structure and function of the human lung to evaluate the lung toxicity of nanoparticles.[24] Liver on chip can be dynamically perfused, and has a lifespan of at least 28 days, which also helps query patient-specific liver response.[25]

However, it is still difficult to quantitatively control collagen fiber orientation *in vitro* and analyze the effect of recombinant collagen tissue on tumor cells. In this study, we constructed a 3D biochip with oriented collagen fiber for spheroid invasion. The orientation of collagen fibers can be quantita-

tive controlled by regulating the concentration and injection rate of collagen, which simulates the micro-environment of the spheroid *in vivo*. The combination of optical microscopic imaging technology to study the invasion of cancer cells, such as the direction of invasion and the speed of invasion, further reflects the significant advantages of biochips in constructing an *in vitro* micro-environment and quantitatively studying invasion.

2. Experimental methods

2.1. Cell culture

Combining several common spheroid culture methods, we inoculated cancer cells into ultra-low attached 96-well plates, so that cancer cells can only grow in suspension, and then form cancer cell spheroids by self-aggregation, and promoted the growth of cancer cells by additives. The human breast cancer cell line MDA-MB-231 (China Infrastructure of Cell Line Resources, Beijing, China) was cultured in complete medium (DMEM, Corning, USA) containing 10.0% fetal bovine serum (FBS, Corning, USA) and 1.0% penicillin/streptomycin (Corning, USA) MDA-MB-231 cells (green) were previously labeled with GFP via stable transfection of DsGFP plasmid vector. Before inoculating MDA-MB-231 cells into 96-well plates, they were grown to 80% fusion in Petri dish (Corning, USA) and washed twice with phosphate buffered saline (PBS, Corning, USA), then digested with 0.25% trypsin-EDTA (Corning, USA) to form a cell suspension and counted by a hemocytometer. The solution was diluted to 2.0×10^4 cells/mL (4000 cells per well). Matrigel (Corning, USA) was thawed in a refrigerator at 4.0 °C overnight, then the liquid matrigel was added to the cell suspension with a frozen pipette, so that the final concentration of matrigel in the cell suspension was 3.5%. A volume of 200 μL of this cell suspension was added to each well of an ultra-low adsorption round bottom 96-well plate (Ultra low attachment, ULA, 7007, Corning, USA). The plate was centrifuged at 1000 Gs (1 Gs = 10^{-4} T) for 10 minutes by using an Eppendorf 5430R desktop high-speed cryogenic centrifuge (Eppendorf, Germany) for the purpose of pelleting the cells at the bottom of the well. Placing the well plate in a constant temperature cell culture incubator with standard cell culture conditions (37.0 °C, 5.0% CO_2, 95% humidity). Finally, the cancer cell spheroids were imaged under an inverted microscope (Ti-E, Nikon, Japan) with a 10X air objective.

2.2. Chip design and preparations

In order to establish an *in vitro* experimental model that is closer to the cancer cell invasion process, we designed and processed a 3D biochip based on polydimethylsiloxane (PDMS) through soft-lithographic technology. As shown in Fig. 2(a), it is an exploded view of the 3D structure of the chip, which is mainly divided into two layers (white), and the upper structure is the cover part, including the injection hole and the outlet hole, used for the injection of spheroid, collagen, and culture medium. The bottom structure is composed of microfluidic channel, collagen channel (blue), and spheroid culture area. Each part is divided by trapezoid pillars (yellow) and the trapezoidal gap of the inner and outer rings is different. The radius of inner ring is 116 μm, while the outer ring 178 μm. The round spheroid culture area in the middle is directly in front of the injection hole of the upper cover. Spheroid is injected into the collagen in this area through the injection hole. When the spheroid culture area is filled with collagen, collagen enters this channel through the gaps of trapezoid pillars. The excess collagen will be discharged to the outside of the chip through the channels on both sides. It has media channels (pink) on both sides, and also through the gaps of the trapezoid pillars (yellow) at the periphery to provide nutrients for the growth and invasion of the middle spheroid. Therefore, the spheroid is restricted to grow in spheroid culture area by trapezoid pillars.

Figure 2(b) is a simulation of the internal flow field of the chip using COMSOL (Comsol Multiphysics 5.4a, Sweden). In the simulation, collagen was injected from the middle injection hole, and the flow rate was reflected by different colors. It can be seen that in the trapezoid pillars, especially the pillars gap between the collagen channel and the spheroid culture area, the flow velocity is the highest, reaching 8×10^{-4} (m/s), which shows there is a significantly high flow velocity when collagen passes through the gap. It will a significant stress field in and around the gap, resulting in *a*-directional orientation of collagen fibers.

As shown in Figs. 2(c1)–2(c3), firstly pure collagen is injected into the center of the spheroid culture area through the middle injection hole, and the mixed solution will form a relatively high flow rate in the gap as shown in Fig. 2(c2). After curing the collagen for 60 min in a 20-°C environment, the collagen fibers will generate a fiber orientation (brown) consistent with the flow field distribution. Secondly the spheroid is injected into the spheroid area [see Fig. 2(c2)]. Then the medium is injected into the medium channel on both sides from the injection holes on both sides [see Fig. 2(c3)]. Finally placing the chip in a cell incubator with a concentration of 5.0% CO_2, a temperature of 37.0 °C, and a saturated humidity of 95%. Light microscopy is applied to obtain cancer cell invasion pictures.

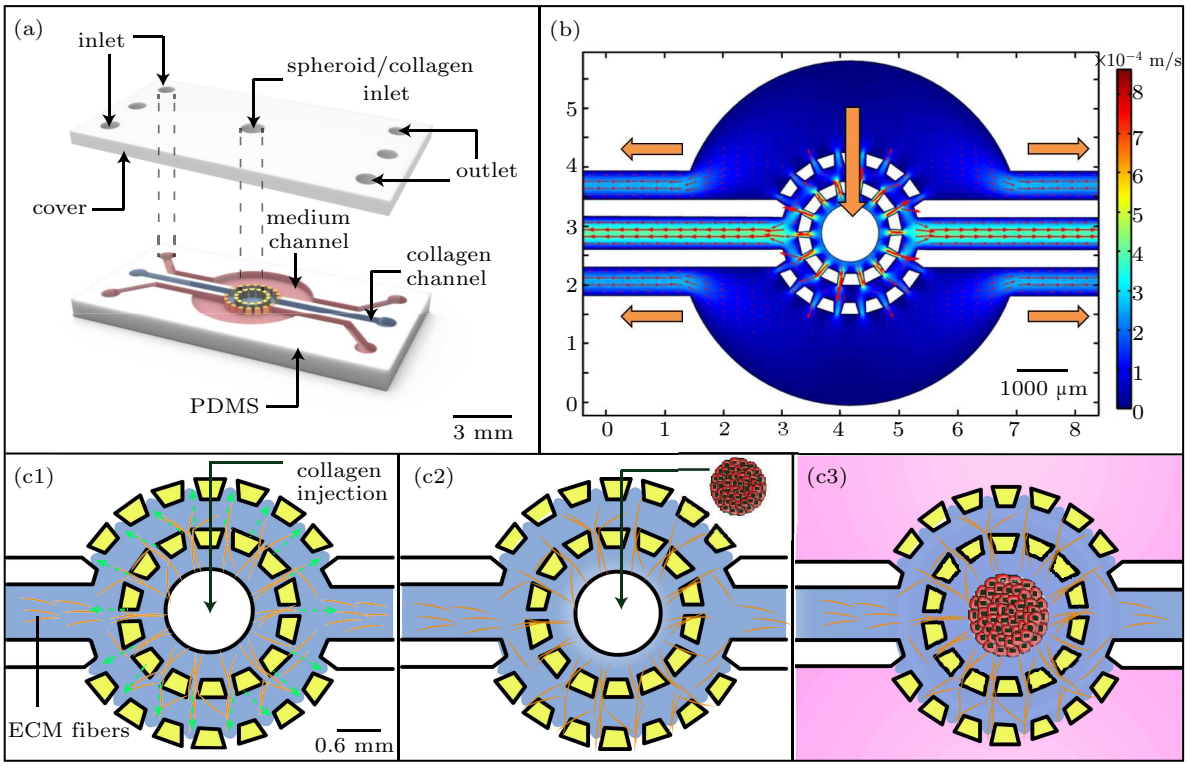


Fig. 2. The microchip design, flow field simulation, and experimental operation. (a) The 3D design of the chip. (b) Simulation result for complete flow field, showing the consistent flow field distribution of the chip. (c1)–(c3) Injection process for the collagen and spheroid (red). (c1) Injecting collagen into the chip from the middle hole (blue channel). (c2) Seeding spheroid into the collagen from the middle hole. (c3) Injecting medium from both channels to provide nutrition for cell invasion (pink channel).

2.3. Analysis of microscopy image

Real-time spheroid compaction and invasion processes were measured using an inverted time-lapse microscope (Ti-E, Nikon, Japan). For compaction analysis, spheroids composed of green fluorescent cancer cells were imaged at 24-h intervals for 72 h. The readout rate of the Nikon microscope is 560 Hz. Its emission wavelength of the laser is 535 nm and the excitation wavelength is 480 nm. The image acquisition of collagen fibers is realized by confocal microscopy (SP8, Leica, Germany). The scan speed of the confocal microscope is 200 Hz, the pinhole size is 53 μm and the emission wavelength is 580 nm. The images were first processed by Curvelet-Based Alignment Analysis Software (University of Wisconsin at Madison) to obtain the angular distribution of collagen fibers, and then statistics and analysis were carried out by Origin (Origin2018, USA). Finally, the Matlab (MATLAB R2015b, USA) helps to obtain the interaction between cancer cells and collagen fibers.

3. Results and discussion

The first experiment performed is spheroid culture and cell invasion analysis in a homogenous collagen ECM environment. The result would be a comparison to the later cell behaviors in oriented collagen in the biochip. In this experiment, 200-μL mixed gel of collagen and cancer cell spheroid (MDA-MB-231-GFP, spheroid) is injected into an ultra-low adsorption 96-well plate, where the concentration of collagen is 2.0 mg/mL. The well plate is placed in a cell culture incubator for cultivation when collagen is solidified. Figure 3(a) shows that the cancer cell spheroids in the well plate for 24 hours taken by reflection pattern (10×) of confocal microscope. The figure shows the cancer cell spheroids (green) and abundant collagen fibers (red) are uniformly distributed in the well plate. The collagen fibers where invading branch occurs exhibit obvious orientation, which is consistent with the direction of the invading branch. However, the collagen fibers at the non-invading branch have no orientation. Figures 3(b1)–3(c1) is an enlarged view (20×) of the confocal image of the invasive branch around the cancer cell spheroid and the non-invasive branch. Figure 3(b1) shows the distribution of cells and collagen fibers in the invading branch, while figure 3(c1) shows the distribution of collagen fibers in the invading branch.

The Curvelet–Based Alignment analysis software was used to analyze the collagen fiber orientation in the B1 and C1 areas around the cancer cell spheroid. The fiber orientation is quantified by measuring its angle relative to the channel direction (x axis).[26] After that, the obtained angle of each collagen fiber is imported into Origin (Origin2017, USA), and the distribution of collagen fibers is obtained through histogram statistics. Here, the horizontal direction to the right x axis is defined as 0°, and the vertical direction is defined as 90°, while

the horizontal direction to the $-x$ axis is defined as 180°. Figure 3(b2) shows the quantitative statistics of collagen fiber angle at panel 3(b1). It shows that the surrounding angles with invasion branches of cancer cells are concentrated at 100°–120° displaying the certain orientation. The localized cell orientation may be resulted by strain field from interactions of spheroid development and collagen fibers. Besides, the cells (green) also shows an orientation similar to that of collagen (arrow), which indicates that the orientation of metastatic cells is closely related to the orientation of fibers. Figure 3(c2) shows that collagen fibers are more randomly distributed in all directions, which means that the orientation of collagen fibers in the orifice plate is more uniform, and it will not significantly affect the spheroid branch.

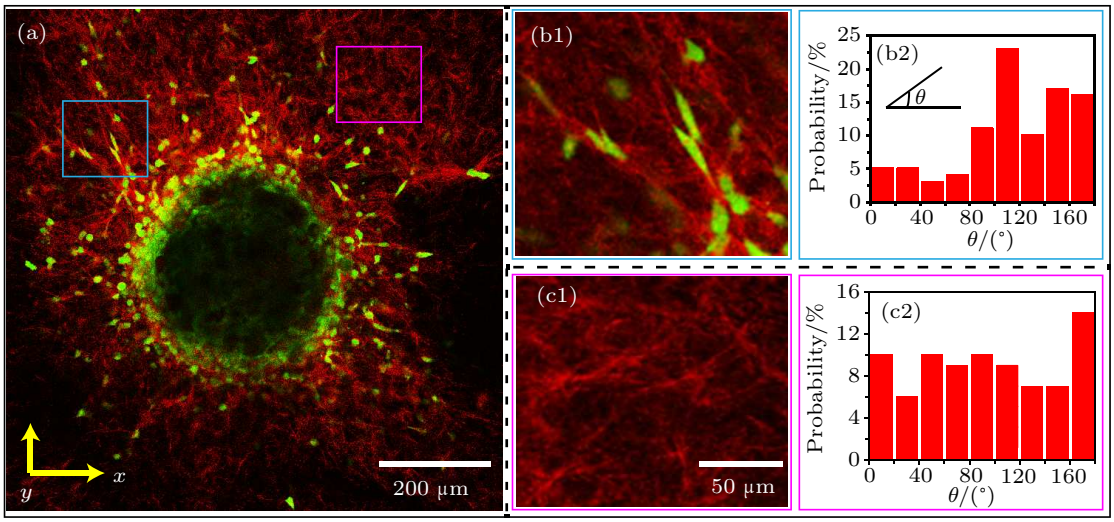


Fig. 3. Orientation distribution of collagen fibers around the three-dimensional invasion experiment of spheroid in 96-well plate. (a) The confocal image of spheroid branches (green) and collagen fibers (red) at 24th hour. (b1) and (c1) The enlarged pictures of the "invasive branches" (blue box) and "non-invasive branches" (pink box) regions, respectively. (b2) and (c2) Statistics of orientation angle of collagen fibers in different parts around spheroid. The orientation distribution analysis of collagen fibers in the "invasive branches" (blue box) and "non-invasive branches" (pink box) regions, respectively.

The extracellular matrix not only serves as an inert support, provides strong and stable support, but also actively participates in controlling cell growth, polarity, shape, migration, and metabolic activities.[27] Collagen is one of the commonly used substances for simulating extracellular matrix *in vitro* and has good biocompatibility.[28,29] Collagen fibers can form a network structure, which can help the growth and invasion of cancer cells through their permeability and orientation.[30,31] At the same time, collagen fibers have good plasticity, and could change their orientation through the stress of the microenvironment, providing some specific microenvironments for the growth and invasion of cancer cells.[32,33]

In order to construct oriented collagen fiber in the chip, we first inject the cancer cell spheroids and collagen in the concentration of 2.0 mg/mL into the middle channel of the chip (2 μL/s) using the syringe pump, and cure in the environment of 20.0 °C for 1 hour. Then injecting the medium from the injection holes on both sides, and placing the chip in the cell incubator for cultivation. At the same time, the culture medium is renewed every 24 hours. Figures 4(a)–4(c) show the spheroid culture area of the chip at 0, 24 (not shown), 48, and 72 hours, the bright-field and fluorescence images of growth and invasion are obtained by inverted microscope. The figure shows the invasion of the cancer cell spheroid at different times. At the 0th hour, the cancer cell spheroid was inoculated into the chip and exhibited no invasion behavior, and the collagen was just solidified. At the 48th hour, the cancer cell spheroid invaded obviously, and the invasion range becomes larger with time. At the 72th hour, apoptosis appeared at the front of the invasive branch, and the invasive range began to decrease. Referring to Figs. 3 and 4, it can be concluded that the oriented collagen fibers have guiding and assisting effects on the invasion of cancer cells.

In order to quantitatively study the changes of cancer cell spheroid invasion over time in the chip, we processed the cancer cell spheroid invasion resulting images obtained from the inverted microscope through the Matlab program. First, the grayscale image is obtained by performing grayscale operation on the fluorescent image. Second, the Gaussian blur algorithm is used to the grayscale image to filter the noise points on the background. Then, the binary threshold of the image is automatically determined through the built-in graythresh function in Matlab, and the image is binarized. Finally, the built-in infill function in Matlab is used to fill the holes in the binary map and determine the largest connected component, as shown in Fig. 4(d1). Here, the processed spheroid boundary is con-

nected to obtain an irregular spheroid, as shown in Fig. 4(d2).

For comparing the quantitative data at different moments, we use a unified polar coordinate system to describe the boundary of the cell spheroid. As shown in Fig. 4(d3), we divide the points on the boundary curve into 300 parts in polar coordinate space, and set the maximum distance in each part to the value of the polar diameter, and polar angle is the radian of the angle bisector in each aliquot. Among them, the distance is defined as the length from the boundary to the center of gravity. After smoothing the edges of the cancer cell spheroid, a new cancer cell spheroid boundary can be obtained. If the shape of the cancer cell spheroid is relatively regular, then the spheroid part is the main part of the cancer cell spheroid, and the branch on the spheroid is the invasive branch. In fact, the shape of the cancer cell spheroid obtained in the experiment is often ellipsoidal, so it is necessary to find the area corresponding to the low frequency part and the high frequency part of the cancer cell spheroid through Fourier transform, that is, the low frequency part corresponds to the nucleus area, the high frequency part corresponds to the attack area.[34]

We have defined several morphological parameters to quantify the invasive characteristics of the spheroid, such as geometric parameters (area, circumference, radius, and volume of the spheroid). Since the invasion branch will make the circumference and area of the cancer cell spheroid relatively increases, we define the ratio of the square of the circumference to the area to quantify the cancer cell spheroid with the invasion branch, and the mutation time of the slope on the curve is defined as the invasion time of cancer cell spheroid. Figure 4(d4) shows the change in the ratio of the square of the spheroid to the area over time (black curve), with a large increase between 0 h and 24 h. The average increase of the ratio of the square of the circumference of the cancer cell spheroid to the area is 16.67, and the slope is 0.69.

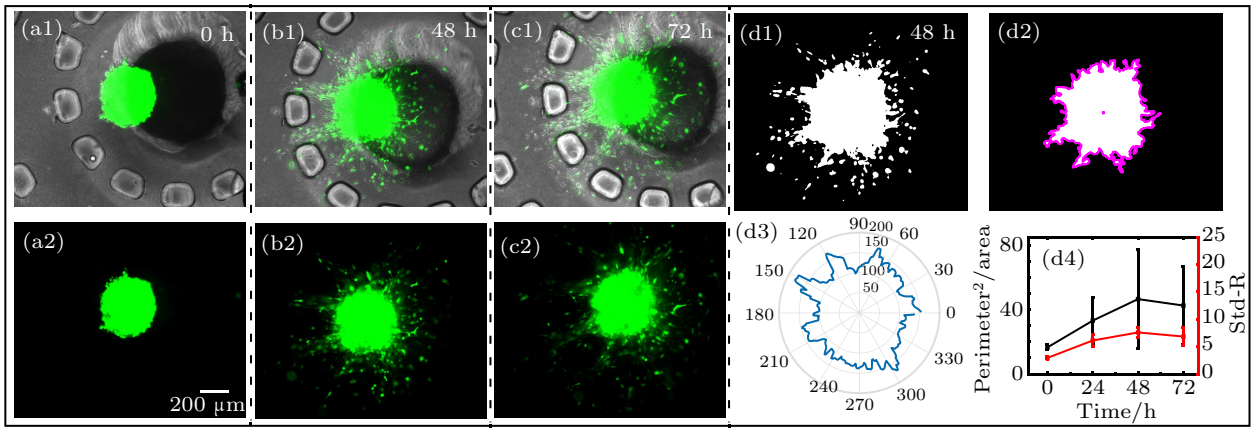


Fig. 4. Experimental diagram of spheroid invasion at different times and data analysis of spheroid invasion in the chip. (a1)–(c1) The fluorescence and bright field merge picture of cancer cells at different times for MDA-MB-231 (green). (a2)–(c2) Experimental invasion of MDA-MB-231 cells at different times. (d) Quantitative treatment of cancer cell invasion. (d1) The binarization of the original fluorescence image. (d2) Boundary connection of image after binarization. (d3) The smoothed boundary is obtained original boundary via boundary smoothing. (d4) The curve of the ratio of the square of the spheroid perimeter to the sphere area with time and the curve of the standard deviation of the distance from the relative boundary to the center with time. From these two trajectories, the transition time can be determined to be 24 h. Scale bar denotes 200 μm.

In addition, the protrusions in the invading branch will also increase the roughness of the cancer cell spheroid, which is characterized by the standard deviation Std-R relative to the boundary to center distance. The results in Fig. 4(d4) (red curve) also show a large increment between 0 and 24 hours. The average increment of Std-R is 3.17, and the slope is 0.13.

We find that the times of the mutation slopes of the two curves are the same, that is, the slope is the largest within 24 hours, indicating that the cancer cell spheroids in the environment of oriented collagen fibers have rapidly invaded. Since the chip can control the orientation of collagen fibers, the two-photon mode (10×) of a confocal microscope can be used to obtain the cancer cell spheroid (green) and the distribution of collagen fibers (white). The image shows that the orientation of collagen fibers and cancer cells is very obvious near the trapezoidal part of the inner circle.

In order to investigate the effect of oriented fiber on cell invasion, we purposefully choose two local areas from the confocal image [see Fig. 5(a)]. These two areas are magnified for details and displayed in Figs. 5(b1) and 5(c1), respectively. Both of these local areas show the detailed fiber morphology, *e.g.*, fiber branch. The Curvelet-Based Alignment Analysis software (University of Wisconsin at Madison) was applied to quantitatively analyze the fiber orientation. Fiber orientation was calculated by measuring its angle relative to the channel direction, and the corresponding angle distributions (red dotted lines) are plotted in Figs. 5(b2) and 5(c2). It is obvious that majority of the fiber distribute around at 0° and 180°, which is parallel to the channel direction, as seen in Fig. 5(c2). Similarly, we also calculated the distribution

of migrating cells based on the cell shape in these two areas, as shown in Figs. 5(b2) and 5(c2). Clearly, the obtained distributions of cell shape behave like these for the corresponding distributions of fiber. In order to quantitatively figure out the relationship between the fibers and the migrating cells, we computed the cross-correlation function defined in the following expression

$$R_{xy}(\tau) = \sum_{n=1}^{N} x(n) \cdot y(n+\tau), \tag{1}$$

where x and y are two discrete signals, τ the corresponding lag between the signals, N the total number of the individual signals.

The results are plotted in Figs. 5(b3) and 5(c3). We find that the values of correlation function at lag = 0, are greater than 0.8 for these two local areas, which indicates the direction of cell invasion is strongly related to the direction of fiber alignment. The results show aligned fibers can direct tumor cell intravasation.

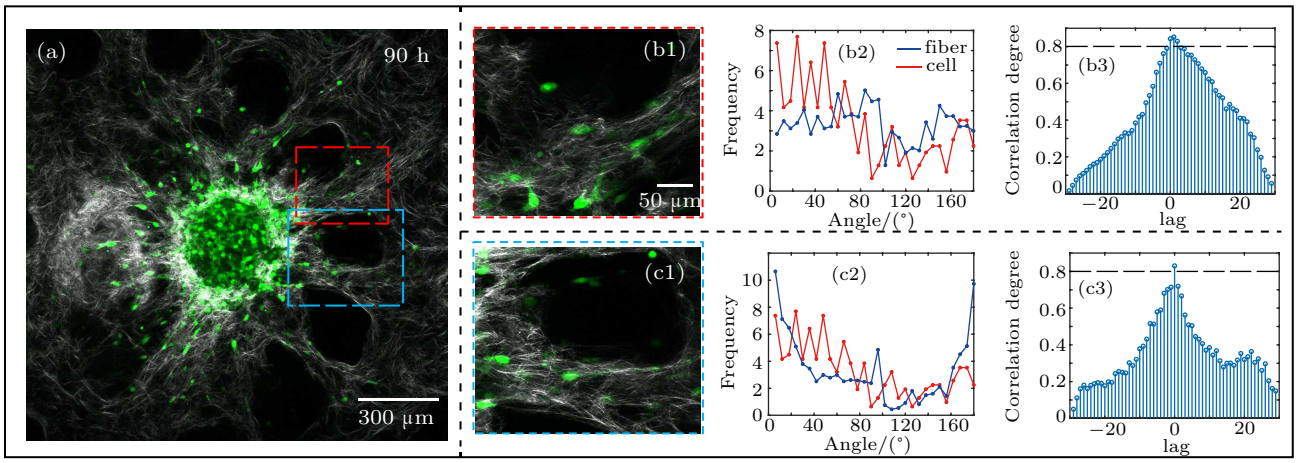


Fig. 5. Analysis of the correlation between spheroid invasion branch cells and fibers in the chip. (a) Confocal image of collagen fibers (white) obtained in two-photon mode of inverted microscope and cancer cells (green) obtained in fluorescent mode at 90th hour. (b1) and (c1) Enlarged views of the invasion branches of two different parts of spheroid, respectively. (b2)–(b3), (c2)–(c3) Representation of the angle distribution of fiber and cell, the correlation between collagen fibers, and cells at the pink box and the blue box, respectively. The Y value is greater than 0.8 at time 0 hour, indicating that collagen fibers have strong correlation with cells.

4. Conclusion

In this study, we designed a 3D chip that can control the orientation of collagen fibers and combined with cancer cell spheroids to study the invasion behavior of cancer cells *in vitro*. We first inoculated cancer cells in a 96-well ultra-low adsorption plate to prevent cancer cells from adhering to the wall, and added 3.5% matrigel as a binder to the cell suspension to promote the formation of cancer cells. Then inoculating the cancer cell spheroids into the targeted collagen fibers, we found that in the environment of oriented collagen fibers, the cancer cell spheroids can quickly and directionally invade within 24 hours, that is, cancer cells can invade along the collagen fibers at extremely high speed. By quantitatively analyzing the invasion of cancer cell spheroids, we found that the invading cells have a strong correlation with collagen fibers. Therefore, we concluded that the tumor microenvironment of collagen fiber orientation plays an important role in guiding cell infiltration and promoting rapid cell invasion.

The results show that the rapid invasion of cancer cells in the organism is not only related to the nutrient gradient, but also closely related to the changes in the surrounding environment. Therefore, we suggest that future treatments may consider adjusting the biophysical microenvironment of the tumor, for example, changing the orientation of already oriented collagen fibers to slow the invasion potential of invasive cancer cells. We can also inoculate epithelial cells on the outermost circle of the chip, which is the outermost trapezoid in Fig. 2(a), forming a dense layer of cells to simulate the blood vessel wall. By directional collagen fibers, cancer cells can invade the outermost trapezium, and by secreting some substances to destroy the blood vessel wall and enter the culture medium channel. In this way, the invasion process that simulates the extravasation of cancer cells into blood vessels *in vitro* is realized in the chip. In addition, we can also add drugs to the media channels on both sides to form a drug gradient to study the inhibitory effect of drugs on the growth and invasion of cancer cells. Therefore, this chip not only provides a model for studying the behavior of cancer cells in a 3D environment *in vitro*, but also build a comprehensive experimental platform for evaluating the effects of drugs.

References

[1] Siegel R L, Miller K D and Jemal A 2019 *CA Cancer J. Clin.* **69** 7
[2] Friedl P, Locker J, Sahai E and Segall J E 2012 *Nat. Cell Biol.* **14** 777
[3] Saxena M and Christofori G 2013 *Mol. Oncol.* **7** 283
[4] Alsarraj J and Hunter K W 2012 *Int. Journal Breast Cancer* **2012** 670632
[5] Hsu Y Y, Chang K W, Chen T S, Lee K Y and Liu C S 2014 *An integrated microfluidic chip for the study of metastasis and angiogenesis* pp. 426–429

[6] Lu J L, Wang X C, Rong X H and Liu L Y 2015 *Acta Phys. Sin.* **64** 058705 (in Chinese)

[7] Hirschhaeuser F, Menne H, Dittfeld C, West J, Mueller-Klieser W and Kunz-Schughart L A 2010 *J. Biotechnol.* **148** 3

[8] Haycock J W 2011 *Methods Mol. Biol.* (Clifton, N.J.) **695** 1

[9] Kimlin L C, Casagrande G and Virador V M 2013 *Mol. Carcinog.* **52** 167

[10] Mayer B, Tischer A, Wieser A, Jauch K W and Funke I 2007 *Mol. Cancer Ther.* **6** 3406S

[11] Markovitz-Bishitz Y, Tauber Y, Afrimzon E, Zurgil N, Sobolev M, Shafran Y, Deutsch A, Howitz S and Deutsch M 2010 *Biomaterials* **31** 8436

[12] Zimmermann M, Box C and Eccles S A 2013 *Methods Mol. Biol.* **986** 227

[13] Chen Z and Zou Y 2018 *Math. Biosci Eng.* **15** 361

[14] Aryasomayajula B 2013 *Dissertations & Theses Gradworks* pp. 195–210

[15] Kurosawa H 2007 *J. Biosci. Bioeng.* **103** 389

[16] Barrila J, Radtke A L, Crabbe A, Sarker S F, Herbst-Kralovetz M M, Ott C M and Nickerson C A 2010 *Nat. Rev. Microbiol.* **8** 791

[17] Vinci M, Gowan S, Boxall F, Patterson L, Zimmermann M, Court W, Lomas C, Mendiola M, Hardisson D and Eccles S A 2012 *BMC Biology* **10** 29

[18] Breslin S and O'Driscoll L 2013 *Drug. Discov. Today* **18** 240

[19] Hu G, Li L and Xu W 2017 *Front. Laboratory Med.* **1** 36

[20] Zhu J, Xiong G, Trinkle C and Xu R 2014 *Histology & Histopathology* **29** 1083

[21] Kaur J and Reinhardt D P 2015 *Stem Cell Biology and Tissue Engineering in Dental Sciences*, Vishwakarma A, *et al.* eds. (Boston: Academic Press) pp. 25–45

[22] Järveläinen H, Sainio A, Koulu M, Wight T N and Penttinen R 2009 *Mathematical Intelligencer* **31** 40

[23] Aguilera K Y, Huang H, Du W, Hagopian M M, Wang Z, Hinz S, Hwang T H, Wang H, Fleming J B, Castrillon D H, Ren X, Ding K and Brekken R A 2017 *Mol. Cancer Ther.* **16** 2473

[24] Zhang M, Xu C, Jiang L and Qin J 2018 *Toxicol. Res. (Camb.)* **7** 1048

[25] Schepers A, Li C, Chhabra A, Seney B T and Bhatia S 2016 *Lab A Chip* **16** 2644

[26] Han W, Chen S, Wei Y, Fan Q and Liu L 2016 *Proc. Natl. Acad. Sci. USA* **113** 11208

[27] Matusiewicz M 2011 *Encyclopedia of Cancer*, Schwab M ed. (Berlin, Heidelberg: Springer Berlin Heidelberg) pp. 1362–1265

[28] Cavo M, Fato M, Peñuela L, Beltrame F, Raiteri R and Scaglione S 2016 *Sci. Rep.* **6** 35367

[29] Sun B 2015 *Acta Phys. Sin.* **64** 058201 (in Chinese)

[30] Tibbitt M W and Anseth K S 2009 *Biotechnol. Bioeng.* **103** 655

[31] Lee B, Konen J, Wilkinson S, Marcus A I and Jiang Y 2017 *Sci. Rep.* **7** 39498

[32] Riching K M, Cox B L, Salick M R, Pehlke C, Riching A S, Ponik S M, Bass B R, Crone W C, Jiang Y, Weaver A M, Eliceiri K W and Keely P J 2014 *Biophys. Journal* **107** 2546

[33] Starke J, Maaser K, Wehrle-Haller B and Friedl P 2013 *Exp. Cell Research* **319** 2424

[34] He Y, Xiong L, Gao X, Hai M, Liu Y, Wang G, Chen G, Shuai J, Jiao Y, Zhang X, Liu R and Liu L 2020 *Biochim. Biophys. Acta (BBA) - Gen. Subjects* **1864** 129460