Chapter 16

3D Breast Cancer Model on Silk Fibroin–Integrated Microfluidic Chips

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Abstract

To imitate in vivo environment of cells, microfluidics offer controllable fashions at micro-scale and enable regulate flow-related parameters precisely, leveraging the current state of 3D systems to 4D level through the inclusion of flow and shear stress. In particular, integrating silk fibroin as an adhering layer with microfluidic chips enables to form more comprehensive and biocompatible network between cells since silk fibroin holds outstanding mechanical and biological properties such as easy processability, biocompatibility, controllable biodegradation, and versatile functionalization. In this chapter, we describe design and fabrication of a microfluidic chip, with silk fibroin–covered microchannels for the formation of 3D structures, such as MCF-7 (human breast cancer) cell spheroids as a model system. All the steps performed here are characterized by surface-sensitive tools and standard tissue culture methods. Overall, this strategy can be easily integrated into various high-tech application areas such as drug delivery systems, regenerative medicine, and tissue engineering in near future.

Key words 3D cell culture, Microfluidics, Cancer, Silk fibroin, Extracellular matrix

1 Introduction

Cancer is a disease with a high mortality rate that affects millions of people annually. Statistical reports, for instance, declared an estimated 19.3 million of new cancer cases and 10 million cancer-related deaths worldwide in 2020 [1]. It is estimated that the expenditures for cancer disease will also rise due to the significant increase in the number of people diagnosed and treated for cancer each year [2]. One critical factor to tackle this overwhelming challenge is the impact of anticancer drug studies. However, anticancer drug development is a complex process involving several costly and time-consuming steps, including drug discovery, drug screening, cytotoxicity studies, preclinical testing, and clinical trials. For a drug compound from bench to market, it takes 10–15 years with a total estimated cost of US$800 million [2]. This complex process demonstrates the urgent need for rapid, affordable, and

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accurate methods for screening and validating potential drug candidates during this entire process. Due to heterogeneity and unstable genetic structure of cancer cells, they constantly mutate and may become resistant to anticancer drugs. One of the problems slowing the development and approval of new anticancer therapies is the paucity of preclinical models that can be used to discover and determine key molecular, cellular, and biophysical assets of human cancer progression.

At the first glance, in vitro studies are taken to assess the interactions and efficacy of a drug to a certain cell type. In traditional cancer research, basic 2D cell culture is employed in which cells form a single layer. As a result, it is not possible to evaluate the spatial interactions maintaining cell-to-cell and cell-to-extracellular matrix (ECM) interfaces that are present in 3D networks of tissues [2]. Despite its advantages such as ease of application and low cost, 2D cell cultures differ noticeably in molecular mechanisms such as gene expression, topology, and biochemistry compared to the in vivo state. This is due to the fact that the majority of in vitro 2D cancer models fall short in accurately recapitulating the complicated pathophysiology of tissue and organ microenvironment, where tumors grow and interact with other cells [3]. Moreover, in vivo studies performed in animal models—as a secondary testing models, also brings many challenges, such as ethical concerns, failing to mimic human tumor physiology, different immunological reaction, and low rate of translatable results into clinical trials [4]. Although they are more expensive and time-consuming, 3D culture systems provide more mimicked environment to in vivo states of tissues [5]. Elaborating with a crucial point, for instance, cells cultured on plastic materials are exposed to nutrient and drug concentrations in a static state, whereas in native conditions, cells experience a 3D matrix environment where blood vessels interact, facilitating the continuous flow of nutrients. [5]. Such demanding points have encouraged researchers to integrate microfluidic systems that are able to tune the flow dynamics and conditions to recapitulate tissue milieu. Accordingly, in past decades, harmonizing microfluidic chips with 3D cell culturing strategy in biological studies has provided unique research realms, such as organ-on-a-chip and disease-on-a-chip, further personalizing the concept into personalized medicine and precision health strategies [6]. The most striking benefits of such devices include low-reagent consumption, high efficiency, the improved precision, short processing time, low-cost, easy-to-control flow parameters, multifaceted characteristics (mixing, separation, enrichment, etc.), and portability [7, 8]. Therefore, microfluidics holds a mainstay role in biomedical research, especially cancer research and anticancer treatment strategies.

From the fabrication perspective, the earliest materials used for microfluidic chips were silicon and glass; yet, recent advances in biomaterials have denoted new materials such as polymeric

substrates, composites or paper. For instance, microfluidic chips produced from polydimethylsiloxane (PDMS), poly-methyl methacrylate (PMMA), and polystyrene materials are frequently employed in research [9]. Despite its easy fabrication, suitable optical transparency, and elastomeric properties, there are some challenges of PDMS-based microfluidic chips including nonspecific and noncontrollable molecule absorption, liquid evaporation, subsequently forming gradient inside the channels and generating internal flow. To tackle such challenges, these factors need to be closely monitored for proper 3D cell culture; however, it is quite labor-intensive and specialization-needed process, hindering its wide adaptation into cell culture studies [10]. Polystyrene, on the other hand, is frequently employed as a substrate in tissue culture flasks due to its hydrophilic structure, biocompatible in terms of cell adhesion, and being less prone to absorption of hydrophobic molecules. In addition, optical transparency, inertness, stiffness, and ease of surface functionalization are the other striking fashions of polystyrene. In this respect, using polystyrene material in cell culture on-chip studies holds significant potential.

In addition, one of the critical items in developing microfluidic chips for cell culturing is to choose a proper and reliable material. There are several criteria that need to be addressed for selecting an ideal biomaterial: (1) processibility by using mild conditions to facilitate the addition of protein or growth factor, (2) support for adhesion and normal function cultivated cells, (3) allowing potential chemical modification of the surface, (4) slow and predictable rates of deterioration for maximizing the functionality of devices, (5) robustness and flexibility, and (6) relative inexpensiveness. One such biomaterial that meets these requirements is silk fibroin (SF)—a fibrous protein that is the main component of silk and spider web [11]. Although the purified form of silk has been mainly employed in textile industry in the past, today SF obtained from the *Bombyx mori* silkworm is utilized as FDA-approved biomaterials in medicine for a wide variety of applications, such as surgery, drug delivery, and tissue engineering [12]. Owing to its biocompatibility, biodegradability, structural stability, and flexible transition between sol-gel states, SF has been also employed in 3D cell culture applications [13]. As providing an ECM-like environment, SF is able to reflect native conditions observed in cellular environment, enabling cell-to-cell and cell-to-ECM interactions. In this chapter, we comprehensively demonstrate the design and fabrication of a microfluidic chip and further modify the microchannels with SF to form a 3D network with MCF-7 cells as a breast cancer cell model. Hence, it is aimed to mimic tumor microenvironments through the integration of microfluidic chips that reflects the flow in the body and creation of native-like environment with an affordable, facile-to-obtain, and easy-to-modify biomaterial.

2 Materials

2.1 Cell Culture Materials

All cell culture materials must be sterile, including solutions and cell culture plastics.

1. MCF-7 cells, a liquid nitrogen (or −80 °C)-stored aliquot.
2. Growth medium: 10% (v/v) fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) with L-glutamine and sodium bicarbonate, without HEPES.
3. Silk fibroin (SF): 1 mg/mL silk fibroin in distilled water.
4. Dulbecco's phosphate-buffered saline (DPBS) without magnesium and calcium.
5. Trypsin-EDTA solution: 0.25% (w/v) trypsin, 0.02% (w/v) EDTA in DPBS without calcium and magnesium, with phenol red.
6. Trypan blue solution: 0.4% (w/v) Trypan blue in DPBS.
7. Cell counting instrument, e.g., hemocytometer.
8. Serologic pipettes, 2 mL, 5 mL, 10 mL, and 25 mL.
9. T75 cell culture flask.
10. Water bath, set to 37 °C.
11. Micropipettors, 100–1000 μL, 10–100 μL, and 0.5–10 μL.
12. Cell scraper.
13. Incubator with 5% of CO_2 and set to 37 °C.
14. Class II laminar flow cabinet.
15. 70% (v/v) of ethanol.
16. 70% (v/v) of methanol.
17. Molecular biology grade water.
18. Centrifuge tubes, 15 mL and 50 mL.
19. Centrifuge.
20. Electronic pipettor.

2.2 Fabrication of Microfluidic Chips

1. Laser cutter, 0.05 mm of sensitivity capable of cutting polymeric materials.
2. Polystyrene, 2 mm thickness.
3. Double-sided adhesive (DSA), 50 μm of thickness. The microfluidic chip includes multilayers (Fig. 1a): (i) the first polystyrene layer (2 mm of thickness) as the first layer, where inlet and outlet ports of the chip is designed; (ii) five layers of DSA films (50 μm of thickness for each), which provide the height of microchannels (250 μm of thickness in total) and they are

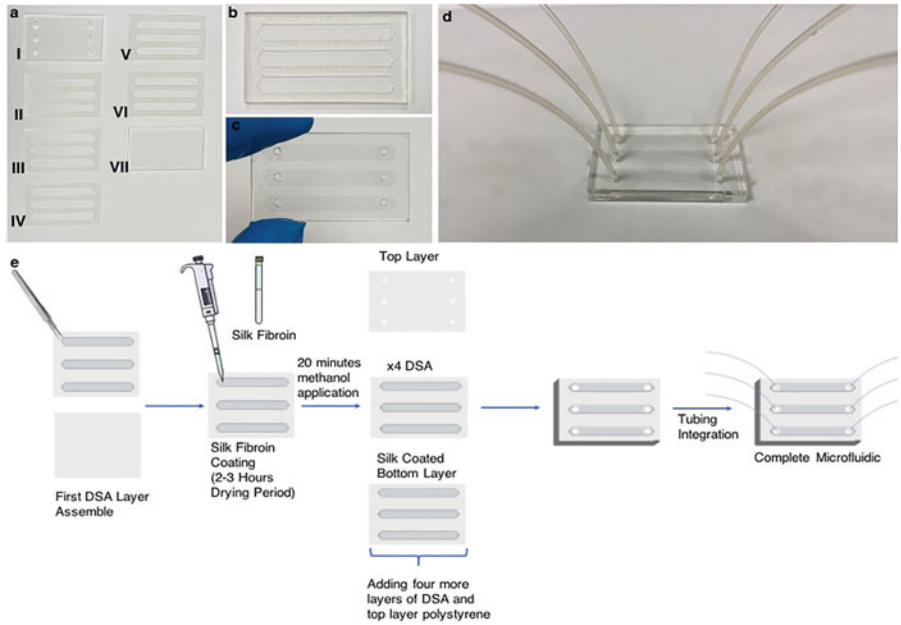

Fig. 1 All the components to fabricate microfluidic chips and the procedure of SF integration to the chips. (**a**) The chip consists of polystyrene materials (I, VII) and DSA films (II-VI). (**b**) Microchannels are formed with the first DSA first. (**c**) SF-coated microfluidic chip. (**d**) Full functional chip is finalized by tubing. (**e**) Schematic representation of all fabrication and modification steps

used to assemble the chip components; and (iii) the second polystyrene layer (2 mm of thickness) as the bottom layer where silk will be coated, and then, cells are grown on it.

4. Poly(tetrafluoroethylene) (PTFE) tubing, inner diameter 0.91 mm, outer diameter 1.46 mm, wall 0.30 mm.
5. Absolute ethanol (100% ethanol).
6. Tweezers.
7. Syringe pump with six channels, flow rates 0.452 μL/h to 1451 mL/h.
8. Sterile syringe, 20 mL.
9. Epoxy resin and hardener.
10. 21 gauge needles.

2.3 3D Cell Culture Imaging; RNA Isolation and Quantification

1. Hoechst 33342 solution: 1 μg/mL Hoechst 33342 in PBS.
2. RNA isolation kit, e.g., GenElute™ Mammalian Total RNA Miniprep Kit.
3. 70% (v/v) ethanol for RNA.
4. RNase-free pipette tips.
5. Molecular biology grade water.

6. Inverted fluorescence microscope, with a brightfield module and four different fluorescent filters
7. Microcentrifuge.
8. Vortex mixer.
9. Spectrophotometer to measure RNA concentration (e.g., Qubit 4 Fluorometer with Qubit™ RNA High Sensitivity assay kits or Nanodrop).

2.4 Materials for cDNA Synthesis, PCR Analysis, and Imaging

1. High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems)
2. PCR tubes.
3. Thermal cycler with an accuracy of ±0.2 °C, uniformity of ±0.4 °C, and temperature range between 0 and 100 °C.
4. Powdered agarose.
5. 1× Tris-acetate-EDTA (TAE) buffer. To prepare the stock (10×) TAE buffer, mix 48.5 g of tris base, 11.4 mL of glacial acetic acid, 20 mL of 0.5 M EDTA (pH 8.0), and add distilled water to reach the volume to 1 L. To prepare 1 L of 1× TAE buffer, mix 900 mL of distilled water with 100 mL of 10 × TAE buffer.
6. SYBR safe DNA gel stain.
7. 5× loading dye: 10% (w/v) SDS, 500 mM DTT, 50% (v/v) glycerol, 250 mM Tris–HCl, 0.5% (w/v) bromophenol blue dye.
8. DNA ladder (1 kb and 50 bp).
9. AmpONE Taq premix (GeneAll).
10. PCR primers for housekeeping gene, e.g., glyceraldehyde-3-phosphate dehydrogenase (GAPDH): GAPDH Forward primer 5′- TCCCTGAGCTGAACGGGAAG -3′, GAPDH Reverse primer 5′- GGAGGAGTGGGTGTCGCTGT-3′.
11. Nuclease-free water.
12. Agarose gel tray and tank.
13. Comb (15 wells).
14. Microwave.
15. Power supply (10–300 V).

3 Methods

All cell culture procedures must be performed in a sterile environment of the laminar flow cabinet (i.e., cell culture hood). Sterilize the laminar flow cabinet with UV for 15 min before starting to culture cells. Once sterilization is over, turn the laminar flow on and

wipe the cabinet work surface with 70% of ethanol. Before use and placement into the laminar flow cabinet, wipe all cell culture flasks, serological pipettes, medium bottles, centrifuge tubes, etc., with 70% of ethanol.

3.1 Cell Culture

1. Pipette 5 mL of growth medium into a 5 mL tube and warm it up in a 37 °C water bath.
2. Remove the MCF-7 cell vial from −80 °C/liquid nitrogen and thaw it at 37 °C in a water bath.
3. Transfer the thawed cell suspension to the 15 mL tube with pre-warmed medium (*see* **Note 1**).
4. Centrifuge the tube at 300 × *g* for 5 min.
5. Discard the supernatant, resuspend the pellet in 5 mL of medium, and transfer it to the T75 cell culture flask.
6. Incubate the cells in a cell culture incubator (5% CO_2, 37 °C).
7. Change the medium of the cells every 2 days. When the cells cover >85% of the flask surface, passage them (*see* **Note 2**).

3.2 Cell Passaging

1. Before passaging, incubate medium and trypsin-EDTA solution at room temperature to allow them to warm up.
2. Check the cells using a microscope, and then, put the cell flask into the cabinet.
3. Aspirate the medium from the cell flask.
4. Wash the cells with 2 mL of PBS (in order to remove any waste, dead cells, and cell debris) twice.
5. Add 1 mL of trypsin-EDTA solution to the cells and incubate the flask in the cell culture incubator at 37 °C for 5 min (*see* **Note 3**).
6. Add 4 mL of fresh medium into the flask (*see* **Note 4**) and pipette the suspension several times.
7. Transfer 1 mL of the cell suspension into a new flask. Add 4 mL of fresh medium into this flask, thereby achieving passaging at a 1:5 sub-culture ratio.
8. Incubate the new flask in an incubator at 5% of CO_2 and 37 °C.

3.3 Fabrication of Microfluidic Chips

1. All the chip parameters are designed with RDWork software; cut all these materials with a laser cutting machine.
2. Cut tubing, which is used for sampling and introducing the medium into the channels, with a razor. The length of the inlet tubing should be 60 cm, and the outlet tubing should be 20 cm.

3. Clean all materials required for fabricating the chip with absolute ethanol and disinfect them under UV of tissue culture cabinet for 1 h (*see* **Note 5**).
4. Adhere a layer of DSA to the bottom base of the microfluidic chip inside the hood after sterilization. Thus, channel formation is provided for the microfluidic chip (Fig. 1b).
5. After the first layer of DSA, introduce 40 μL of SF into the channels. Keep the microfluidic chip in the hood for 2–3 h to allow SF to dry (*see* **Note 6**).
6. Introduce 40 μL of 70% methanol into the channels and incubate for 20 min to minimize SF from dissolving in water (*see* **Note 7**).
7. Wash the layer of SF with a sterile medium or PBS.
8. Complete fabricating a microfluidic device by assembling the other four DSA layers and the top layer of (first) polystyrene (*see* **Note 8**) (Fig. 1c).
9. As the final step of the chip fabrication, fix tubing into inlet and outlet ports of microfluidic chips with epoxy (*see* **Note 9**) (Fig. 1d, e).

3.4 Cell Cultivation in Microfluidic Chips and Imaging

1. Trypsinize the cells as described in Subheading 3.2, **steps 1–6**.
2. Count the cells: In a microcentrifuge tube, mix 10 μL of cell suspension with 10 μL of Trypan blue. Using a hemocytometer, count the number of unstained (white-looking) cells under a microscope (dead cells are observed in blue color).
3. Calculate the number of viable cells per mL.
4. Introduce the number of cells required for an experiment (e.g., 50,000–70,000 cells per channel) into the microchannels through the inlet tubing (*see* **Note 10**).
5. After seeding cells, keep the microfluidic chip in an incubator (5% of CO_2, 37 °C) for 24 h, so that cells adhere to the surface of the chip.
6. Next day, connect the inlet tubing to a syringe pump while the microfluidic chip is inside the incubator and introduce fresh growth medium to these cells with a continuous flow (2 μL/min) using a syringe pump, which is located outside the incubator (Fig. 2) (*see* **Note 11).**
7. Take images daily with a microscope. After 4 days of culture on-chip, 3D structures should be visible inside the microchannels (Fig. 3).
8. To stain the nuclei, apply 100 μL of Hoechst 33342 solution into the microfluidic chips and incubate for 10 min, protected from light.

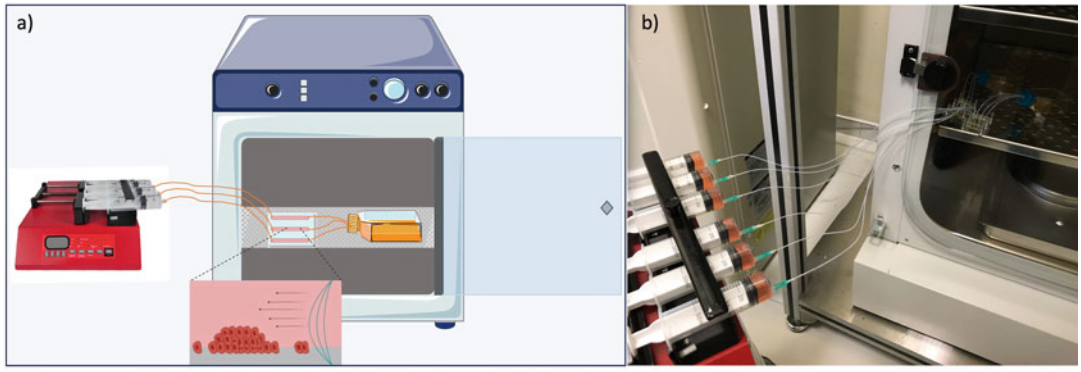

Fig. 2 A microfluidic chip operated with a syringe pump is employed to culture cells inside an incubator. (**a**) Schematic representation and (**b**) real experimental setup are exhibited. For scaling-up, the experiment can be operated with multiple channels of a chip and/or multiple microfluidic chips at the same time. In this representative experimental set, cell culture is performed in two separate microfluidic chips simultaneously

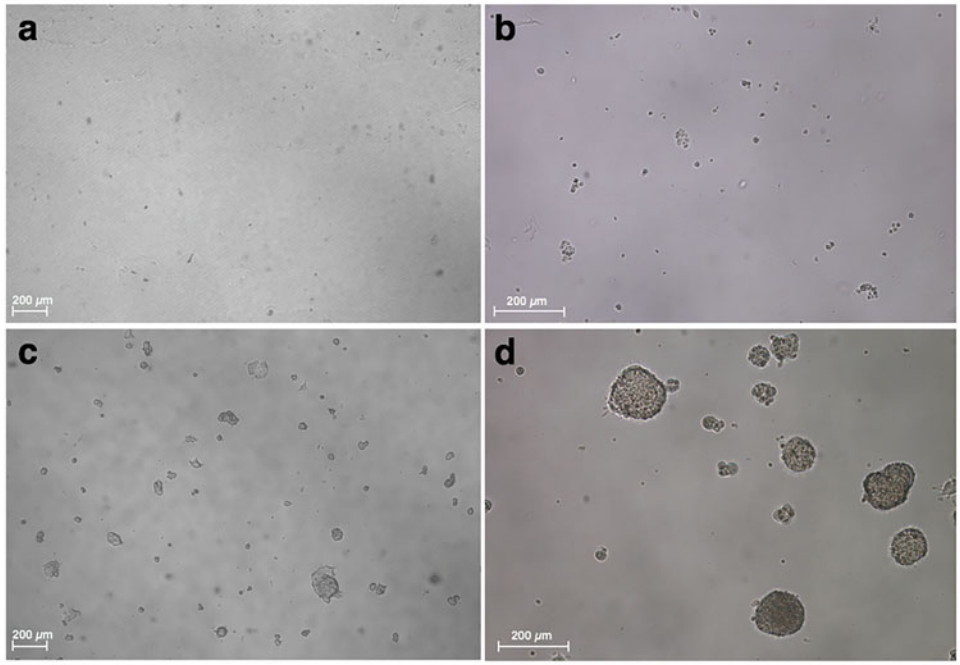

Fig. 3 Cells are grown on SF-coated microfluidic chips. (**a**) The image of the microfluidic channel coated with SF before cell seeding is presented. (**b**–**d**) The images show day 2, 4, and 7 of cell culture. From the day 4, cells start to form 3D spheroid networks. By the day 7, these structures remain intact and vary in size. Scale bars, 200 μm

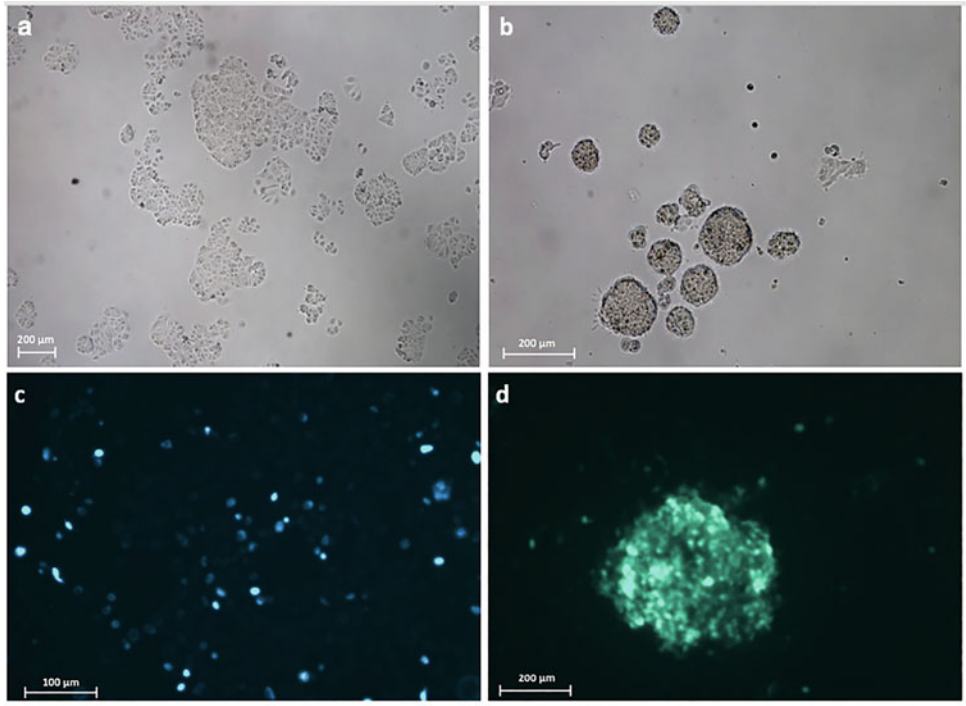

Fig. 4 Morphologies of MCF-7 cells cultured in 2D and 3D. (**a**) Brightfield image shows the monolayer of MCF-7 cells growing in 2D culture flask. Scale bar, 200 µm. (**b**) Brightfield image presents the spheroid structures of MCF-7 cells growing in a microfluidic chip. Scale bar, 200 µm. (**c**) Hoechst 33342-stained cells in 2D culture. Scale bar, 100 µm. (**d**) Hoechst 33342-stained cells in a microfluidic chip. Scale bar, 200 µm

9. Using a syringe, introduce 400 µL of PBS into the microfluidic chips at a rate of 2 µL/min to remove the dye from the chip. Repeat three times.
10. Visualize the cells stained with Hoechst 33342 dye using a microscope with fluorescent filters (excitation/emission values of 350 nm/461 nm) (Fig. 4).

3.5 RNA Isolation and Quantification

1. After 7 days of culture, harvest cells from microfluidic chips by applying trypsin-EDTA solution to the microchannels. First, add 3 mL of fresh growth medium into a centrifuge tube in which the cells will be collected and place the outlet tubing of the microfluidic chip in this tube (*see* **Note 12**).
2. Apply trypsin-EDTA solution into the microchannels through a syringe pump for 5 min at 200 µL/min of flow rate.
3. Centrifuge the cells harvested at 300 × *g* for 5 min.
4. Discard the supernatant and add 500 µL of lysis buffer with 1% (v/v) 2-mercaptoethanol to the pellet.
5. Vortex until any clumps disappear.

6. Place the filtration column in a 2 mL collection tube. Apply the suspension of lysed cells to the filtration column and centrifuge at 12,000 × *g* for 2 min.
7. Add 500 μL of 70% ethanol into the filtered lysate and mix well using a vortex or a micropipette.
8. Place a binding column in a new 2 mL collection tube. Apply 700 μL of lysate-ethanol mixture to the binding column and centrifuge at 12,000 × *g* for 15 s.
9. Discard the flow-through and apply the rest of lysate-ethanol mixture to the binding column. Centrifuge at 12,000 × *g* for 15 s.
10. Discard the flow-through and add 500 μL of Wash Solution I into the binding column and centrifuge at 12,000 × *g* for 15 s.
11. Transfer the binding column into a new 2 mL collection tube. Add 500 μL of Wash Solution II into the binding column and centrifuge at 12,000 g for 15 s.
12. Discard the flow-through. Add 500 μL of Wash Solution II into the binding column and centrifuge at 12,000 g for 2 min (*see* **Note 13**).
13. Transfer the binding column into a new 2 mL collection tube and add 50 μL of elution solution to the binding column. Centrifuge at 12,000 × *g* for 1 min (*see* **Note 14**).
14. To quantify RNA, use Qubit™ RNA High Sensitivity assay kit. Prepare the working solution by diluting RNA HS reagent in RNA HS buffer at the ratio 1:200.
15. Calibrate the Qubit instrument as per the manufacturer's protocol. In two separate tubes, mix (i) 190 μL of working solution with 10 μL of standard 1, and (ii) 190 μL of working solution with 10 μL of standard 2, and mix these solutions well (*see* **Note 15**).
16. Read the tube with standard 1 mix (i), and then read the tube with standard 2 mix (ii).
17. To quantify RNA amount in the sample, combine 198 μL of working solution and 2 μL of RNA sample in a tube, mix well, and read the tube in the instrument.

3.6 cDNA Synthesis; PCR Analysis and Imaging

This protocol uses High-Capacity cDNA Reverse Transcription Kit. Other kits can be used to synthesize cDNA.

1. Prepare the reverse transcription master by combining 2 μL of 10× RT buffer solution, 0.8 μL of 25× dNTP mix, 2 μL of 10× RT random primers, 1 μL of reverse transcriptase, and 4.2 μL of nuclease-free water well in a tube.
2. Add 10 μL of the master mix and 10 μL of RNA sample to a PCR tube (*see* **Note 16**).

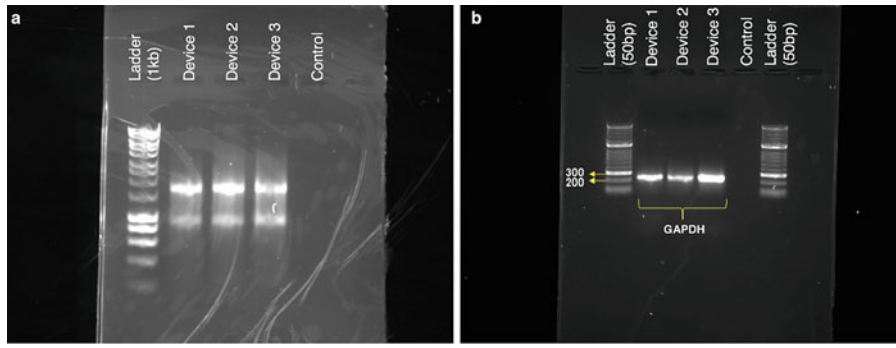

Fig. 5 Gel images are obtained after cDNA synthesis and PCR analysis. (**a**) The synthesis of cDNA indicates that the enough amount of RNA is isolated from the cells grown in microfluidic chips. (**b**) The GAPDH (housekeeping gene) expression has been confirmed with cDNA. According to the forward and reverse primers of GAPDH, the band would have been around 220 bp. The band shown in the results matches the expected value

3. Place the PCR tube in the thermal cycler to start cDNA synthesis.
4. Program the thermal cycler to 10 min at 25 °C, 120 min at 37 °C, 5 min at 85 °C, and cooling to 4 °C.
5. Optional: Perform an agarose gel electrophoresis to confirm cDNA synthesis (Fig. 5a).
6. Perform PCR using housekeeping gene to normalize the levels of mRNA among different samples (Fig. 5b). Mix 1 μL of GAPDH Forward primer, 1 μL of GAPDH Reverse primer, 2 μL of cDNA sample, and 16 μL nuclease-free water to the Taq premix strip.
7. Place sample tubes in the thermal cycler. Set the PCR program to: 95 °C for 2 min, [95 °C for 20 s, 60 °C for 10 s, 72 °C for 20 s] repeated for 35 cycles, 72 °C for 5 min, 4–12 °C for 5 min.
8. When the PCR is finished, perform agarose gel electrophoresis.

3.7 Gel Electrophoresis

1. Prepare 1% of agarose gel by adding 1 gram of powdered agarose into 100 mL of TAE buffer solution. Heat the mixture using a microwave for 1–2 min (*see* **Note 17**).
2. Allow agarose gel to cool down, add 2 μL of SYBR Safe DNA, and pour it into the gel tray.
3. Insert a comb into the poured gel and allow the gel to solidify.
4. Prepare DNA for loading by mixing 1 μL of 5× Loading dye with 4 μL of DNA sample (e.g., cDNA after RT-PCR, or a PCR product).
5. Load the DNA samples onto the gel and run at 90 V for 30 min (*see* **Note 18**).
6. Visualize the DNA using UV illumination and an imaging device.

4 Notes

1. After thawing, cells need to be centrifuged immediately, and DMSO (a cryoprotectant) needs to be removed from cells after the centrifugation. If the cells are exposed to DMSO for a long time, toxic effects may be observed, and accordingly, cell viability may be hindered.
2. It takes several days for the cells to reach 85% confluence, depending on the initial number of the cells seeded.
3. If all the cells are not detached from the surface of cell culture flask after 5 min, the attached cell can be detached with a cell scraper. It is necessary to apply slow movements while detaching the cells from the surface with a cell scraper. If the cell scraper is used too fast, the cells can break down.
4. Medium should be added in at least twice the volume of trypsin-EDTA solution in order to neutralize trypsin activity.
5. DSA films and SF do not need UV sterilization. SF is obtained in sterile condition, and all sample preparation steps for SF took place inside the tissue culture hood. In addition, high and prolonged exposure of UV may alter the structure of SF, and hence, there should not be any UV sterilization step for SF. On the other hand, DSA films may be affected if they are not UV-resistant. Since DSA films are compatible with organic solvents, it is sufficient to wipe the DSA films with absolute ethanol.
6. It is recommended to work between 2 °C and 10 °C while preparing the working concentration of SF. Moreover, vortexing and rapid pipetting would potentially lead to the formation of β-sheets in the structure. Therefore, working concentration should be prepared as slowly as possible. After adding SF to the microchannels, it should be checked whether the SF has spread throughout the microchannel evenly.
7. Once SF dries, the microfluidic chip is placed in a petri dish, and the lid is closed to prevent methanol from volatilizing. Therefore, for 20 min, the methanol will stay on the SF without evaporation.
8. SF should be allowed to dry in the hood for a while after washing with PBS or a clean medium. If the other DSA layers are added before the channels are completely dry, these layers are affected by wetness and cannot adhere together effectively.
9. It is necessary to place the tubing in the inlet and the outlet one at a time and fix them in an order. If all tubing is placed and fixed simultaneously, some may be dislodged so that epoxy may

fill the inlet and outlet ports; therefore, the channels may be blocked. To prevent this issue, the tubing should be placed in order, and the other tubing should be fixed after the epoxy dries.

10. To cultivate the desired number of cells in the channels, it is necessary to know the volume of tubing used; because, some of them remain in the tubing while the cells are introduced into the microchannels. As an alternative, cells can be introduced directly into the channels before integrating the tubing with the chip, and the tubing can be fixed to the chip with epoxy treatment later.
11. High flow rates should be avoided when cells are inside the chip, since it may cause the detachment of cells from the surface. In addition, the appropriate flow rate should be calculated according to the dimensions of the channel.
12. The cells are collected in the centrifuge tube containing fresh growth medium to quickly neutralize trypsin, thereby preventing the cells from any damage due to a long incubation with the enzyme.
13. The binding column must be utterly free of ethanol before the isolated RNA can be eluted. To ensure this, the binding column can be centrifuged for 1 min at the highest speed ($16{,}000 \times g$).
14. If the expected RNA yield is greater than 50 μg, another 50 μL of Elution Solution should added to the column and centrifuged for 1 min with both eluates collected in the same tube.
15. During the calibration, the value displayed on the screen should be compared with the values of the standards. If the calibration is not accomplished properly, the amount of RNA will not be accurate.
16. Tubes are centrifuged briefly to ensure that the sample is mixed well, and also in order to remove any air bubbles.
17. After the agarose is added to TAE buffer, it is microwaved to dissolve. It is necessary to run the microwave only for about 30 s, then remove the beaker containing the solution, and shake it gently for several turns. This process is repeated several times until the agarose dissolves completely. Care should be taken to not overboil the agarose.
18. Depending on the power source used, the running time of the agarose gel may vary. Therefore, the gel should be checked at intervals of 10 min. Hence, the separation process is completed before the sample comes out of the gel.

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