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Mathématiques, Sciences de l'Information et de la Communication (MSTIC)

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Spécialité : Electronique, Optronique et Systèmes

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Fu Yi

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Conception, Fabrication et Expérimentation de Systèmes Microfluidiques de Culture Cellulaire pour la Recherche sur le Cancer et la Neurobiologie

Design, Fabrication and experiment of Microfluidic Cell Culture for Cancer and Neurobiology Research

Directeurs de thèse

Tarik BOUROUINA et Ai-Qun LIU

Jury

Vincent SENEZ, Directeur de Recherche, IEMN CNRS-Université de Lille1	Rapporteur
Olivier FRANÇAIS, Maître de Conférences, SATIE, CNRS-ENS Cachan	Rapporteur
Karima BRAHIMI, Docteur, Institut PASTEUR et VAC-4-ALL	Examinateur
Gaëlle LISSORGUES, Professeur, ESYCOM, ESIEE Paris	Examinateur
Ai-Qun LIU, Professeur, Nanyang Technological University, Singapour	Examinateur
Tarik BOUROUINA, Professeur, ESYCOM, ESIEE Paris	Examinateur

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SUMMARY

In this PhD project, two *in vitro* cell culture devices were developed via microfbrication technologies, which provided entirely new levels of controls over the cell culture microenvironment. The applications of the developed devices in cancer and neurobiology researches were demonstrated, specifically for the fundamental study of cancer metastasis and neural axonal pathfinding.

The microfluidic transmigration chip used microchannel structures to mimic the tissue capillaries along the path of cancer cell metastasis. The transparent optical qualities of the device allowed good observation of the deformation and migration of cells in the artificial capillaries. The influences of geometric confinement on cell migration were studied using microchannels of varying dimensions. Results showed that deformation of the stiff cell nucleus were the most time-consuming steps during the transmigration process. The physical restrictions not only changed the morphology of the cells, but also significantly affect their migration profile.

Further studies on the molecular contents and biological properties of the transmigrated cells showed that the mechanical stress imposed on the cancer cells by the confined geometries could induce biochemical modifications on their nuclear histone proteins and the elevations of their transmigration capabilities. Blocking the histone modifications by specific drug can inhibit the transmigration of cancer cells in the microchannel, which might have implications on cancer prevention and treatment. The microfluidic chip can also be used to evaluate cell deformability, which is a potential prognostic marker for cancer diagnosis.

The neural culture chip integrated microfluidic cell culture and protein patterning techniques. The somas and axons of neurons cultured in the device can be polarized into different fluidically isolated environments for long period, and the extension of the axons can be guided by proteins immobilized on the glass substrate into specific patterns. The oriented axon growth can be further modulated by localized drug treatment. Studies on the underlying molecular mechanism revealed that these processes were closely associated with the proteins synthesized locally in the tips of growing axons.

In conclusion, the capabilities of microfabricated cell culture devices in mimicking complicated *in vivo* cellular microenvironments were demonstrated through two novel devices for cancer and neuroscience researches. With the new techniques and research methodologies available, more fundamental questions regarding the structures and functions of individual cells can be addressed and it will lead to a deeper understanding of many basic biological processes and better medical treatments of related diseases.

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CHAPTER 1

INTRODUCTION

1.1 Motivation

Mimicking the extracellular environments through engineering approaches enables biologists to culture cells *in vitro* and study the interactions between cells and environments, which are important for the proper development and function of cells, and also for the pathogenesis of many diseases [1-7]. The heterogeneous extracellular environmental cues cover large spatial and temporal scales, ranging from individual molecules to entire organisms. Due to technical limitations, most of the traditional *in vitro* cell culture platforms are only capable of presenting single environmental cues ubiquitously and homogenously to large groups of cells. Although numerous researches have been done, information on the responses to multiple extracellular signals at single cell or sub-cellular level are limited, and the studies on the molecular mechanism behind these biological events are also difficult to be performed using traditional methods.

Advanced researches in various fields of molecular and cell biology require new devices to be developed for better mimicking of the microenvironments encountered by individual cells when they grow *in vivo*. For example, devices with microstructures whose dimensions and mechanical properties are comparable to the tissue capillaries will be useful for the studies on the metastasis of cancer cells from their original place to distant sites [8]. And in neurobiology, studying how neural cells navigate over long

distances to seek targets and establish connections also requires devices which are able to present groups of different guidance cues to developing neurons [9].

Microfabrication techniques were originally developed for the fabrication of silicon-based microelectronic devices. When used in the development of cell-based microdevices, they are able to create either two dimensional (2D) or three dimensional (3D) structures with micrometer-scale precision, and incorporate different materials with distinct chemical or physical properties [10-20]. The microenviromental factors within the structures can also be precisely controlled. These advantages make the micro-fabricated cell culture devices suitable candidates to solve the existing problems in cancer and neurobiology research.

1.2 Objectives

The main objectives of this PhD project are to develop new *in vitro* cancer and neural cell culture devices via microfabrication techniques. The new devices will be capable of mimicking cellular microenvironment at single cell or sub-cellular level. The developed devices will be used to study the functional effects of various extracellular signals on the growth and development of cancer and neural cells. Toward these goals, series of works will be conducted with the following perspectives:

1. To develop microfabricated devices with micrometer-scale structures mimicking the confined geometries and the fluidic conditions in the extracellular environments of cancer cells. The devices will be used for the study of cancer cell transmigrations, which are critical steps during their metastasis process. The

effects of some important environmental factors on the transmigration profiles of the cancer cells will be systematically studied.

2. To perform different molecular and cell biology assays on the transmigrated cancer cells, study whether the drastic changes of cellular shape during transmigration are associated with the changes in their chemical components and biological properties. If these changes do happen and play critical roles in the transmigration process, pharmacological approaches aiming to inhibit the changes will be explored.
3. To develop microfluidic neural culture device which is able to present multiple environmental cues to specific sub-cellular domains of developing neural cells. Study the functional consequences and the molecular mechanism of these signals on the guidance of neural axon growth.

The detailed research works will include theoretical analysis, establishment of the fabrication processes and experimental protocols, and analysis of the data obtained through the new devices and research methodologies.

1.3 Major contributions

The major contributions of this PhD thesis are summarized as follows:

1. A polydimethylsiloxane (PDMS) based microfluidic transmigration device was developed. It used microchannel structures to mimic the tissue capillaries along the path of cancer cell metastasis. The transparent optical qualities of the device allowed good observation of the deformation and migration of cells in the

artificial capillaries. The influences of geometric confinement on cell migration were studied using microchannels of varying dimensions. Results showed that deformation of the stiff cell nucleus were the most time-consuming steps during the transmigration process. The physical restrictions not only changed the morphology of the cells, but also significantly affected their migration profile. Drastic cellular deformations in the narrow microchannel greatly reduces amount of cells migrate through the microchannels and their velocities. It could not be compensated by the increase of chemoattractant which provided directional cues and driven power for migration.

2. The consequences of physical deformation on the chemical contents and biological properties of the transmigrated cancer cells were studied. Through series of experiments, it was proved that the mechanical stress imposed on the cancer cells by the confined geometry during transmigration could induce biochemical modifications on their nuclear histone proteins. Histone modification resulted in the increase of cancer cells' transmigration capabilities as they could migrate through the narrow microchannels easier than those non-modified cells. Blocking the histone modifications by specific chemicals could inhibit the transmigration of cancer cells in the microchannels, which might have implications on the designing of new anti-cancer therapy.
3. An integrated chip for *in vitro* culture of neural cells was developed, the somas and axons of neurons cultured on the chips could be polarized into different fluidically isolated environments for long period, and meanwhile the extension of the axons could be guided by proteins immobilized on the glass substrate into

specific patterns. The orientated axon growth can be further modulated by localized drug treatment. Studies on the underlying molecular mechanism revealed that these processes were closely associated with the proteins synthesized locally in the tips of growing axons.

1.4 Organization of the thesis

The thesis is organized into six chapters. The introduction of the thesis covers the motivation, objective, and major contributions of the thesis as presented in this chapter. The motivation section explained why PhD research is carried out. The objectives state the main focus of this thesis, and the contribution section lists the innovations and important findings.

In Chapter 2, a literature survey on the background knowledge related to this PhD project is presented. It covers the introduction of microfabricated cell culture devices, summaries of their key features and commonly used fabrication techniques. Information on cancer disease and neural cell development will also be provided, followed by overview of current methodologies used in researches. The existing problems which can be possibly solved with microfabrication technologies will be discussed.

In Chapter 3, a microfluidic chip for the study of cancer cell transmigration is presented. Firstly, design and working principles of the chip are demonstrated. The fabrication process for the chip and the protocols for transmigration experiments are then elaborated, followed by the experimental results. Several different types of cancer cells with distinct metastatic capabilities are tested on the chip, the effects of different

geometry designs and experimental conditions on the transmigration profiles of the cancer cells are studied. Deformation of cancer cells in restricting microchannel during transmigration is also characterized at single-cell level and the critical steps in this process are determined.

In Chapter 4, further analysis are performed on the transmigrated cancer cells to explore if the mechanical stress from the physical environment will induce changes in the chemical or biological properties of the cancer cells. To study whether the drastic changes of nucleus shape during transmigration are associated with alterations of nuclear chromatin structure, nuclear components of cancer cells are analyzed by both real-time observation during the transmigration process and end-point immunofluorescence assay with antibodies recognizing specifically modified histone proteins. Transmigrated cancer cells are isolated from the microchips and propagated into standard cell culture devices. Daughter cells harvested from propagation are also analyzed to check if they can inherit the chemical and biological properties of the mother cells. The possibility of using chromatin-modulating drugs to prevent cancer metastasis is then explored. Specific drugs with capabilities of blocking chromatin structural changes are applied to the cancer cells and their transmigration profiles are studied.

In Chapter 5, a neural culture chip with integrated microfluidic cell culture and protein patterning techniques is presented. Firstly, design and working principles of the chip are illustrated. The fabrication methods for the device and the experimental protocols are then elaborated, followed by the experimental results. Compartmentalized culture of embryonic mouse cortical neurons is demonstrated. The guidance effect of the immobilized protein patterns on the growth of neuron axons is studied. The molecular

mechanism behind the orientated axon growth is also explored through localized drug treatment.

Chapter 6 concludes and summarizes the major contributions of this thesis followed by the recommendations for future works.

CHAPTER 2
LITRERATURE SURVEY

In this chapter, background information related to this PhD project is reviewed. The chapter consists of three parts, the first part is about microfluidic technologies and their applications in biology research, including the introduction of basic concepts, summary of the some environmental factors which are critical for cell culture in the microfluidic chips, and a brief introduction of the commonly used fabrication techniques. The second and third part introduce two cell biology research fields in which the microfluidic techniques will be used to solve scientific research problems. Part two is about cancer biology, including the basic knowledge of cancer disease, tumor cells and their metastasis process, the existing methodologies used in the research and their limitations are also briefly reviewed. Part three is about neurobiology, mainly focus on the development of neural cells and the methodologies used in the research. At last, the existing problems for current researches are discussed and the possible solutions with microfabrication technologies are proposed.

2.1 Microfluidic cell culture chips

2.1.1 Overview

Microfluid specifically refers to the handling of liquid at microliter (μL) or smaller volume in the microchips. Due to the high hydrostatic resistance, surface tension and the friction forces from the confined geometry structure, the liquid inside the microfluidic chips are either static or flow at a much lower velocity than in macroscale devices. Under these situations, lamina flow and diffusion become the dominant effect for mass transportation [21].

The microfluidic chips have some important advantages over the traditional devices. With versatile fabrication techniques such as lithography, etching and surface modification , plus the abundant available materials with different chemical or physical properties such as polymers, metals, and dielectric materials, both 2D and 3D structures with micrometer and sub-micrometer dimension can be constructed within the chips, which are close to the dimension of single cells [22, 23].

The micro-enviromental factors such as the mechanical strength, topology of the substrate, the medium composition, and the fluidic conditions in the microfluidic chips can be precisely controlled [24]. Cellular responses to multiple extracellular stimuli presented at single cell or sub-cellular level, which are often overlooked in traditional cell-based devices, can be systematically studied [25-27]. The microfluidic chips consume much less samples and reagents than traditional devices, therefore reduce the cost of research. Meanwhile multiple experiments can be performed simultaneously thus high throughput experiments can be achieved [28-30].

2.1.2 Microfluidic chip and cell culture

Fluidics and mass transportation

In the microfluidic chips, the dominant effects of fluids change from the macroscale to the microscale. Lamina flow and diffusion are the dominant effect for mass transportation [21]. Laminar flow is characterized by a low Reynolds number which is normally less than 2300. The Reynolds number of a flow can be expressed as:

$$Re = \frac{\rho V D}{\mu} \tag{2.1}$$

where ρ is the fluid density, μ is the fluid viscosity, D is the hydraulic diameter, and V is the characteristic velocity. Due to the narrow hydraulic diameter (small D value), flow in microscale devices is usually laminar and viscous forces dominate. Laminar flow in microchannels allows the partial treatment of single cells, which is difficult to be achieved on traditional macroscale cell culture device [31].

Convection and diffusion are the two major mechanisms for particle transportation in liquid. The transportation of a particle can be quantified using the Peclet number, which is expressed as:

$$Pe = \frac{v l}{D} \tag{2.2}$$

where v is the velocity, l is the characteristic length, and D is the diffusion coefficient of the particle. For large Peclet numbers, convection dominates, while slower velocities and shorter lengths cause diffusion to dominate [32]. Particles diffuse from regions of higher concentration to regions of lower concentration and their flux can be represented mathematically with Fick's first law of diffusion, which can be expressed as:

$$J = -D\frac{dC}{dx} \tag{2.3}$$

where J is the flux of particles, D is the diffusion coefficient, C is the concentration and x is the position. The mean distance d of a particle with diffusion coefficient D diffuses in time t, can be described with equation:

$$d^2 = 2Dt \tag{2.4}$$

Diffusion becomes the dominant transport mechanism only at long time scales and short distances. Diffusion is the main mode of transport in microscale cell culture systems but not for macroscale systems [33].

In macroscale cell culture, stirring is commonly used to distribute metabolites and minimize waste accumulation. Mixing at the microscale is usually difficult to achieve, resulting in less homogenous distributions of metabolites and waste products [34, 35]. Although this might be adverse for cell growth in some cases, the microfluidic cell culture chips can be used to study the accumulation and depletion of cellular waste, metabolites, and other factors such as growth factors, hormones, cytokines and lipids within certain microenvironment [36, 37]. Concentration gradients of biomolecules can be also created through controlled diffusions, which are important for the study of some biological processes like cell migration and orientated cell growth [38-41].

Mechanical stress

Cells grow *in vivo* are constantly subjected to various kinds of mechanical forces from the microenvironments. For example, cells exposed to liquid flow, such as endothelia cells (ECs) in blood vessels, are subjected to shear stresses [42-44]. For *in*

vitro studies in the microfluidic chips, laminar flow in the microchannel structures can impose shear stresses to the cells grow on the channel walls. When the flow speed is precisely controlled through specific chip design, dynamic range of shear stresses, either constant or pulsatile, can be generated in the microdevices [45-47]. Another way to impose mechanical forces on cells is through physical deformation, the cells can be deformed by fluid forces or the microstructures restraining the cells, such as microchannels, posts and sieves [48, 49].

The mechanical forces imposed on cells are transmitted from cell surfaces to their interior components, including cytoplasm, nucleus, and focal adhesion sites, through the structural framework of cytoskeleton. The mechanical stimuli can be eventually translated into intracellular signals that affect cellular functions such as proliferation, apoptosis, migration, permeability, and remodeling, as well as gene expression [50-54].

Material interfaces

Complicated inner structures in the microfluidic chips result in high surface area to volume (SAV) ratios. Material interfaces exist between the cells and their immediate solid (e.g. walls of microchannels) and liquid (e.g. cell culture medium) surroundings, as well as between the culture medium and the geometry structures in the chips. They play important roles in microscale cell culture.

Attachment of cells onto the material interfaces in the cell culture chips can influence many cellular characteristics, including adhesion strength, orientation, cytoskeletal structure, and their growth profile [55]. Typically cells adhere to substrates via focal adhesion complexes (FACs), which are combinations of different adhesive proteins on the cell surface. Binding to different substrates require different combinations of FACs, and different compositions of FAC proteins have different effects on cytoskeletal structures to which they connect [56]. In the microfluidic cell culture chips, micropatterning techniques can be used to geometrically define where specific cells will adhere to a particular substrate, and their adhesion responses to different substrates can be systematically studied [57, 58].

Surface adsorption refers to the phenomenon of molecules superficially adhering to a surface. Hydrophobic macromolecules (e.g. proteins, lipids) in the cell culture medium can be attracted to nearby hydrophobic surfaces in the cell culture chips, cause the depletion of nutrients in culture medium and subsequently affect cell culture conditions [59]. In macroscale culture, the relatively small surface area does not cause drastic alterations of medium composition, while in microscale culture conditions, the

high SAV ratios can cause significant depletion problem. This need to be take in to consideration for the design of microfluidic cell culture chips. Technically the depletion problems can be reduce through surface modification.

2.1.3 Fabrication techniques

Soft lithography

The fabrication techniques used for the production of microfluidic chips was originally developed in the semiconductor industry for integrated circuit production. Classically, the techniques have been used in the productions of microelectronic systems, micromachines, micro-optical systems and their subfields [60-64]. In recent years, these techniques have been used to create cell-based microdevices. Most of the traditional fabrication techniques use silicon or glass as the construction materials, and the fabrication need to be performed in expensive cleanroom facilities. In addition, silicon is not transparent, which limits its applications in the studies requiring high-resolution imaging. Therefore, a faster, less expensive, and less specialized method for device fabrication was needed in order to promote the use of microchips in biology.

Soft lithography is a common, easy, and useful technique to fabricate biologically relevant structures with dimensions from a few nanometers to hundreds of microns. The soft lithographic fabrication process begins in a similar manner to silicon fabrication for semiconductor, with a layer of photoresist material deposited onto a flat surface and patterned with light. The photoresist is developed into a three dimensional negative master, from which a curable polymer mold can be cast. This polymer, commonly PDMS

(polydimethylsiloxane), is then used either directly as a structural component of the device, or is used to pattern another substance onto a surface. The final products of soft lithography is a slab or membrane of PDMS, or occasionally some other polymer with similar properties [65]. Typical process flow of soft lithography is shown in Fig. 2.1, it consists of several major steps including: a) coating, b) lithography, c) pouring/curing and d) plasma bonding.

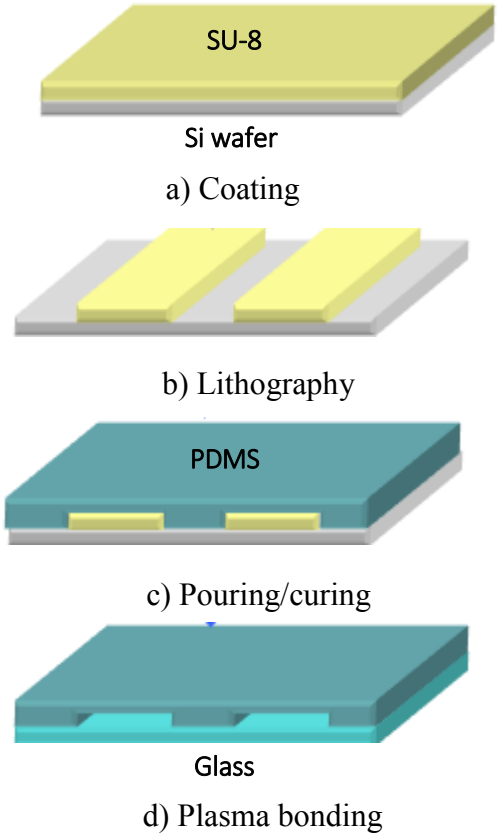


Figure 2.1: Typical soft lithography process flow

Enhancement of biocompatibility through surface modification

The biocompatibility of microfluidic chips can be enhanced through different surface modification techniques including plasma deposition, irradiation, chemical vapor deposition (CVD), covalent modification, and protein immobilization. Plasma is a mixture of electrons, neutral radicals and ions with high energy, in which positive and negative charges are present in equal amounts. Sources of plasma include glow discharges, radio frequencies, and gas arcs. Plasma deposition usually render the treated surface highly hydrophilic, which are favorable for the attachment and growth of cells [66].

Irradiation can generate free radicals, which act as sites for graft polymerization. Ultraviolet (UV) and gamma irradiation are two commonly used methods. Biocompatible polymers, such as acrylic acid (AA), acrylamide (AM) and poly (ethylene glycol) monomethoxyl acrylate (PEG) can be easily grafted on irradiated surface [67, 68]. The other way to immobilize biomolecules on the microfabricated surfaces is through covalent bonding. Immobilization of cell-adhesive proteins or oligopeptides on the surface can also enhance cell adhesion [69, 70]. Chemical vapor deposition (CVD) is a process which transforms gaseous molecules into a solid material which can form a thin film on the surface of a substrate. Silicon nitride (Si3N4) deposited through plasma enhanced chemical vapor deposition (PECVD) or low pressure chemical vapor deposition (LPCVD) is a good material for cell attachment. The PECVD method has the advantage of a low deposition temperature and relatively fast process while the LPCVD technique is

a high throughput technology. The main reason for enhanced cell attachment is the presentation of NH_2 groups on the thin film surface [71, 72].

Surface patterning

Mimicking the complicated extracellular microenvironment requires the technologies to pattern multiple, bioactive molecules on the surfaces which support cell growth. For this purpose, several different surface patterning techniques have been developed to pattern either hydrophilic or hydrophobic compounds onto the surface of silicon, glass or polymer with micrometer-scale precision for cell attachment and growth [73]. Among all the developed techniques, microcontact printing (uCP) has been widely used as a powerful method for patterning relatively fragile, environmentally sensitive compounds such as proteins onto the surface of different substrate. In this method the elastomer stamps containing raised regions of desired patterns are coated with proteins of interest, and placed in direct contact with the working surface. The molecular pattern is thus transferred to the working surface in this process, and remains after separation of the stamp from the substrate as shown in Fig. 2.2 [74, 75].

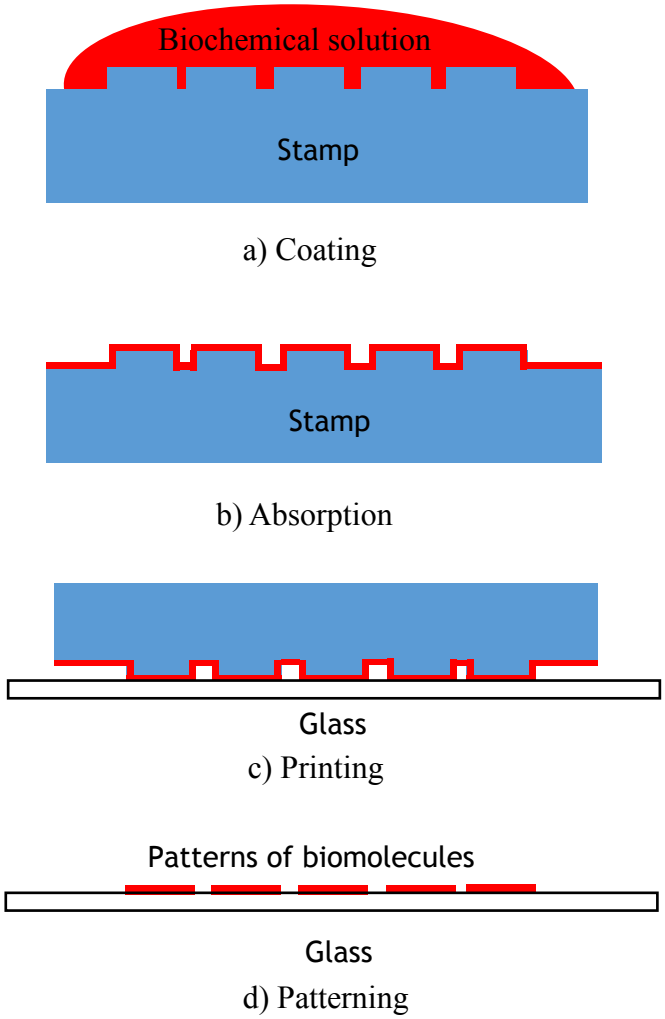


Figure 2.2: Processes flow of microcontact printing

2.2 Metastasis and transmigration of cancer cells

2.2.1 Cancer cell metastasis and transmigration

Cancer is a broad group of diseases in which cells divide and grow uncontrollably, form malignant tumors, and invade nearby parts of the body. It occurs from genetic damage on a single cell in tissue which will result in uncontrolled cell proliferation. This uncontrolled proliferation, known as mitosis, can produce primary heterogeneic tumor. The cells in the heterogenetic tumor undergo series of transformation, including metaplasia, dysplasia and anaplasia, eventually change to malignant phenotype. Drastic phenotypic and biochemical changes occur during the metamorphosis of a normal tissue cell into an invasive cancer cell [76].

The main threat and the reason for most cancer deaths are not the primary neoplasias but secondary tumors, spread from their original organs to other non-adjacent organs or body parts. As shown in Fig. 2.3, during the development of cancer disease, some cancer cells acquire the ability to penetrate the walls of lymphatic or blood vessels, after which they are able to circulate through the bloodstream to other sites and tissues in the body. After the tumor cells come to rest at another site, they re-penetrate the vessel or walls and continue to multiply, eventually forming another clinically detectable tumor. The new tumors are known as metastatic or secondary tumors. Metastasis is one of the hallmarks of the malignancy of cancer disease [77-79].

When entering (intravasation) and exiting (extravasation) the vascular vessel, cancer cells need to transmigrate across the endothelial layer and the underlying

basement membranes of the vessel walls. The stiffness and geometry confinement of the cellular barriers bring lots of challenges to the cancer cells, which require them to sustain drastic deformations during the transmigration process as shown in Fig. 2.3 [80, 81].

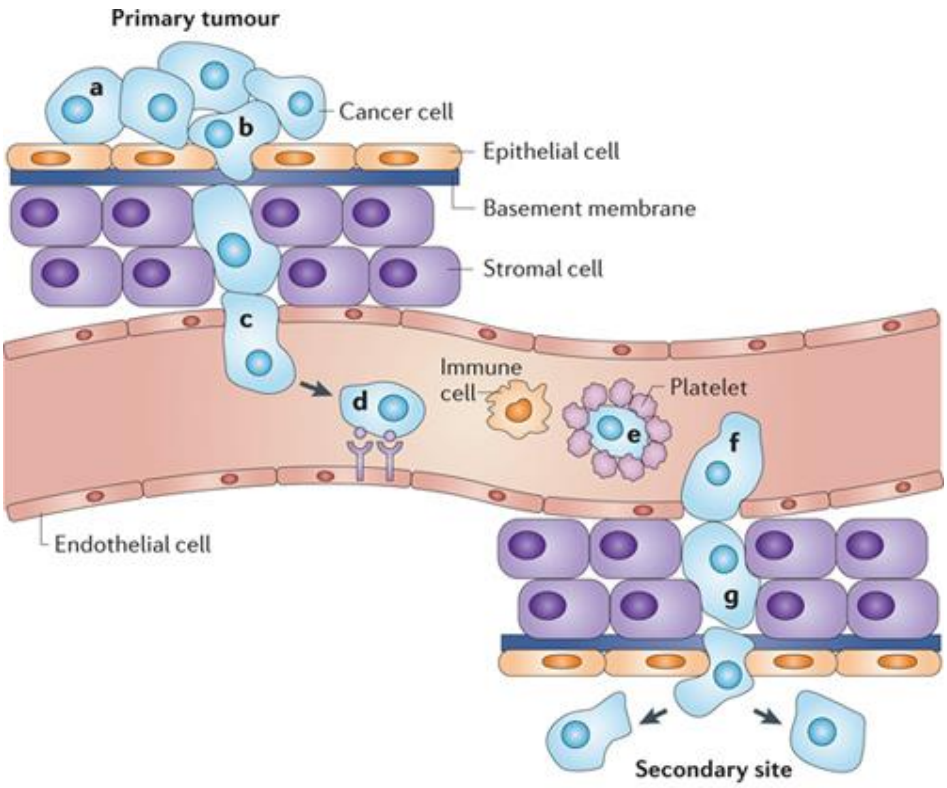


Figure 2.3: Cancer cell metastasis and transmigration (adapted from reference [79]).

As shown in Fig. 2.4, the cell body consists of the plasma membrane surrounding gel-like cytoplasm containing small organelles with dimensions below 1 μm and cytoskeletal filaments. These structures are morphologically adaptable to vigorous

changes and able to adjust to any shape. In contrast, the nucleus is the largest cellular organelle which consists of dense genetic materials surrounded by a lipid bilayer envelope and supported by a network of structural proteins. The nucleus is approximately 5–10 times stiffer than the surrounding cytoskeleton. Therefore it commonly resists large changes in shape [82]. Consequently, for migration through small pores or 3D scaffolds, the nucleus can become the rate-limiting organelle. Although previous studies on cancer metastasis have shown that the nucleus might be the rate-limiting organelle, the dynamics of the cancer cell and its nucleus during transmigration has not been characterized and

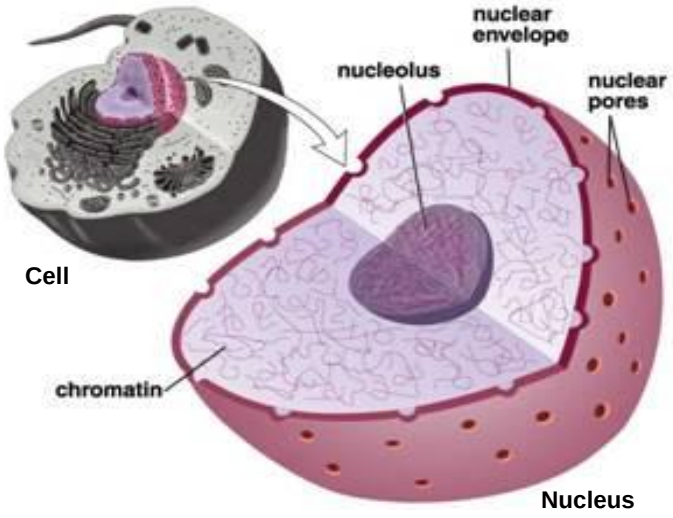


Figure 2.4: Components of a cancer cell and its nucleus (adapted from reference [84]).

their importance remained unascertained [83, 84].

2.2.2 Cell mechanotransduction

Mechanical forces have many effects on the structure and function of cells. The mechanisms by which cells convert mechanical stimuli into different cellular activities are still under extensive researches. Most of the current studies focus on the cells which are involved in sensing and physiological processes such as neurons, and cardiac, skeletal, muscular myocytes [85-88]. The basic concept of mechanotransduction is to convert mechanical signals into intracellular electrical or chemical signals. In cell mechanotransduction process, the extracellular stimulation causes mechanically sensitive ion channels on cell membranes to open and produce a transduction current that changes the membrane potential of the cell [89, 90]. The mechanical stress induced signals can simultaneous activate multiple second messenger systems, which involve many cellular enzymes such as protein phosphatase, kinase and others. Cascade of biochemical reactions will then be triggered in cells by the second messengers and at the far end of the response network, they can affect components in cell nucleus and regulate the level of gene transcription. The cellular responses to mechanical stress are rapid and reversible, which can be reflected on cellular mRNA level [91-93].

Cancer cells sustain drastic mechanical stress when the cells travel through the confined geometries along the path of their metastasis. Studies on the response of cancer cells to mechanical stress are much limited as compared with neurons and myocytes. Most of the previous research works focus on the physical consequence of the mechanical stimuli (cellular deformation and force generation etc.) [94, 95], how the

mechanical signals are translated into cellular biochemical events are not clear. In this PhD project molecular mechanism underlying the transmigration of cancer cells will be studied using microfluidic cell culture chip.

2.2.3 Methods for the study of cancer cell transmigration

Despite its importance, cancer cell transmigration is a difficult process to study. One important reason is that cancer cell transmigration occurs in complicated environments which cannot be easily observed by live cell microscopic imaging. Several methods have been developed to mimic the microenviroment for the *in vitro* study of transmigration. Fig. 2.5 shows the Boyden chamber assay which widely used in which

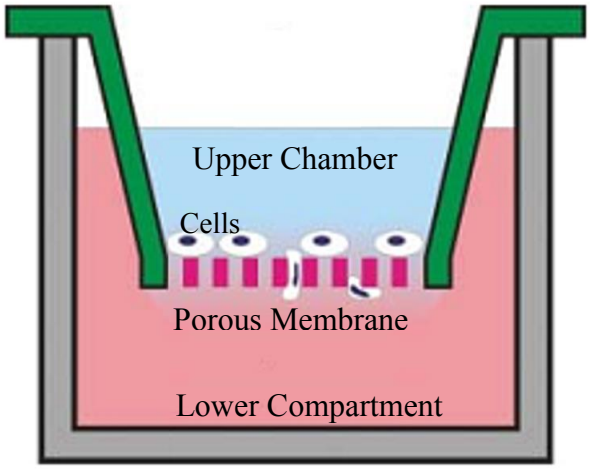


Figure 2.5: Working principle of Boyden chamber (adapted from reference [98]).

cells migrate across a filter towards a passive chemotactic gradient [96, 97].

While much knowledge has been gained from the *in vitro* assays such as Boyden chambers and other membrane or gel based migration devices [98, 99], these methods have several major drawbacks which make them unsuitable for cell migration dynamics study. Firstly they are relatively bulky and the migration events occur far from the surface observable for microscopic imaging, thus they are primarily end-point assays and cannot be used for live cell imaging. Secondly, these systems rely on uncontrolled chemoattractant gradients to induce migration, the gradients dissipate over time which make the experimental parameter difficult to be precisely controlled. For membrane based migration device such as Boyden chamber, the diameters of the pores on a single device are usually identical. Therefore, to study the effect of pore dimensions on the cell migration profile, microfluidic chips containing microchannels of different dimensions will be developed in this PhD project to overcome the shortcomings of the existing devices.

2.3 Neural cells and orientation of axon growth

2.3.1 Neural culture and axon guidance

Neurons, commonly known as nerve cells, are special kinds of cells which are able to connect to each other and form neural networks. Neurons are electrically excitable and able to processes and transmit information through electrical and chemical signals within the networks. Some major subtypes of neuron include sensory neurons which respond to stimuli affecting the cells of the sensory organs, then send signals to the brain, motor neurons which receive signals from the brain and spinal cord and subsequently cause muscle contractions, and interneurons which form neuron-neuron connections in the brain. They are the core components of the nervous system [100].

As shown in Fig. 2.6, the basic structure of a typical neuron consists of one cell body (soma), one axon and several dendrites. Dendrites are thin structures that originated from the cell body, usually extending for hundreds of micrometres and branching multiple times, eventually form complex structures known as “dendritic tree”. Axons are also cellular extension arise from the cell body like dendrites but they are able to travel for much longer distance, some can be as far as hundreds or thousands times than the length of dendrites. In the majority of the cases, axon of one neuron contact the dendrite of another to form special connection named synapse and send electric or chemical signals through the connections.

Axons and dendrites do not appear in young neurons, they arise from soma during the maturation of neurons and the formation of neural networks. Since axons often need to extend significant distances to their synaptic targets, the mechanism in which

intracellular and extracellular factors coordinate to guide the growth of axons along the designated paths remains hot research topics in neuroscience research.

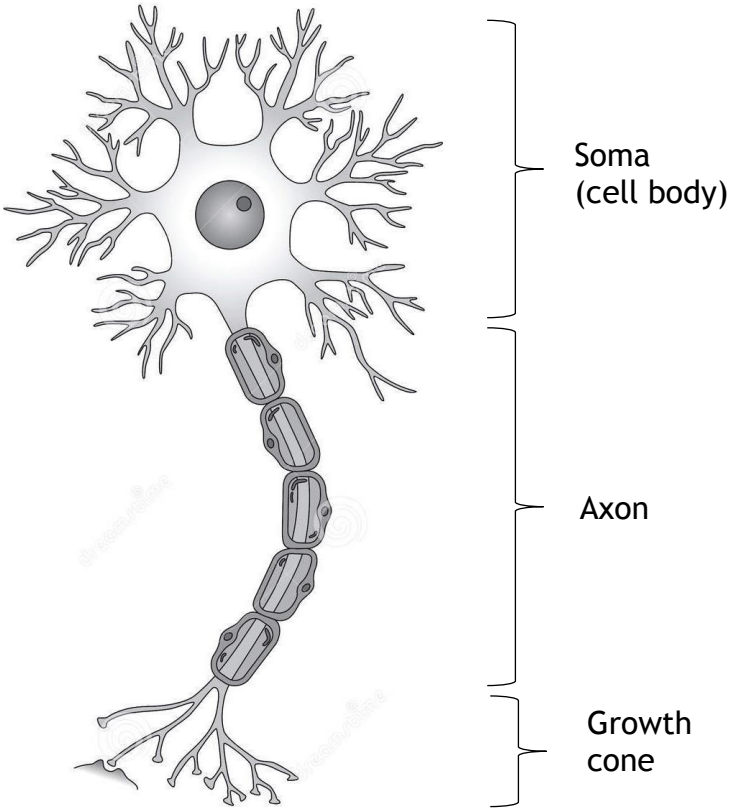


Figure 2.6: Structure of a typical neuron (adapted from reference [102]).

The date most identified extracellular guidance signals are biochemical molecules which can be either fixed in place or diffusible. They are recognized by receptors on the growth cone, a highly motile structure at the tip of growing axons and interpreted into chemotropic responses. These biochemicals may either repel or attract growth cones

and a given receptor may transduce either type of signal, depending on the cellular context. The general mechanism is that the receptors activate various intracellular signaling pathways in the growth cone which eventually affect the cytoskeleton when the growth cone senses a guidance cue. When the guidance molecules are presented in a gradient form, the intracellular signaling in the growth cone happens asymmetrically, so that cytoskeletal changes happen asymmetrically and the growth cone turns toward or away from the guidance cue [101].

Growing axons rely on a variety of guidance molecules when deciding upon a growth pathway. Important classes of axon guidance molecules included cell adhesion molecules (CAMs), developmental morphogens, extracellular matrix proteins, growth factors and neurotransmitters. The cues can be functionally subdivided into adhesive cues which provide physical interaction with the substrate necessary for axon protrusion, tropic cues which can act as attractants or repellents and cause changes in growth cone motility by acting on the cytoskeleton through intracellular signaling, and modulatory cues which influence the sensitivity of growth cones to certain guidance cues. These signals can be further combined and modulated either directly at the cell surface or by the network of intracellular signaling pathways. The results are sophisticated and enable the growth cones to modulate their responses to changing environmental cues along their pathway and successfully extend to their destinations [102-108].

2.3.2 Methods for axon guidance and compartmentalized neural culture

Orientation of axon growth

For the study of axon guidance and construction of defined neural networks, different technologies have been developed to produce gradients of either soluble or substrate-bound guidance cues *in vitro*. These methods provide free soluble gradients of guidance molecules, which mainly rely on the either controlled release or diffusion of the biomolecules in the culture medium. One standard technique is micropipetting. The pipette is filled with a biomolecule solution and positioned at a set distance from cells. The biomolecule solution is pneumatically ejected out of the pipette into the extracellular environment. The frequency of the puffs and the volume ejected with each puff are determined by the pneumatic pump frequency and driving pressure. The puffs combine to form a diffusive gradient that emanates radially from the pipette tip [109]. The other methods which are able to form soluble gradient includes biological hydrogel and some other diffusion based devices [110-112].

For the production of substrate-bound gradients of guidance cues, the biomolecules can be deposited to substrates which support neuron growth using different substrate patterning methods. The strip assay allows substrates to be patterned by depositing different solutions of guidance molecules in separate lanes using a microfabricated silicon or PDMS matix attached to the substrate [113-115]. The other commonly used method patterning method microcontact printing which have been briefly introduced in section § 2.1.3.

While these established methods have achieved remarkable insights into axonal guidance and neural developments, it should be noticed that the extracellular environments supporting neural cells grow *in vivo* are complicated. As most of the existing methods are only capable of presenting single guidance cue, the information on the joint guidance effects of multiple guidance signals are still limited.

Compartmentalized neural culture

Since the neurites, especially axons can extend for long distance from soma to the target sites, neural soma and their neurites often reside in different chemical, physical, and fluidic microenvironments. Discovering how local subcellular signals influence the growth and function of individual neurons requires *in vitro* culture devices which are able to compartmentalize soma and neurites into isolated environments.

The most representative traditional compartmentalized neuron culture systems are Campenot chambers [116, 117], which consist of millimeter-scale barriers that isolate axons and cell bodies into separate compartments. Recently microfabricated versions of the Campenot chamber have been developed, which provide higher reproducibility and better compatibility with a broad range of neurons by eliminating the need for a sealant compound between the chamber and substrate through which neurons must extend their axons [14, 118, 119]. The Campenot chambers have been widely used to study local signaling by restricting the activity of pharmacological agents to distinct parts of the neuron. This allows localized stimulation of receptors on certain part of cell surface and limits the effects of toxic drugs to the rest of the cell [120-122].

2.4 Summary

In this chapter, literature surveys are performed on microfluidic cell culture for cancer and neuroscience research. Firstly, transmigration and metastasis of cancer cells are complicated processes, on which the *in vitro* studies require cell culture devices to mimic the extracellular environment, especially the confined geometries which the cancer cells encounter along the path of metastasis.

Secondly, the development of functional neural networks depends on the ability of neural axons to navigate a complex landscape of guidance cues in the extracellular environment. Due to the lack of suitable research platform, the information on the combined effects of multiple guidance cues are limited. Novel neural culture devices which are able to present both soluble and substrate-bound guidance signals to specific sub-cellular domain of the growing neurons are therefore required.

The microfluidic cell culture chips have many important advantages over the traditional cell culture devices. Most importantly the micro-environmental factors such as mechanical strength, topology, medium composition and fluidic conditions in the chips can be precisely controlled. Newly developed devices with these important features may be able to solve the existing problems in cancer and neuroscience researches.

CHAPTER 3

MICROFLUIDIC CANCER CELL CULTURE CHIP FOR TRANSMIGRATION AND METASTASIS

Transmigrations of cancer cells through confined spaces along the paths of their metastasis are highly complicated process. The dynamic interactions between the extracellular environment and intracellular components can change the physical, chemical and biological properties of the cancer cells. The mechanism underlying these important biological processes are not well understood due to the lack of suitable platforms and research methodologies.

In this chapter, a microfluidic cell culture chip for the study of cancer cell transmigration is presented. Firstly, the microfluidic chip is designed and fabricated, the protocols for cancer cell transmigration experiments are then elaborated, followed by the experimental results. Different types of cancer cells with distinct metastatic capabilities are tested on the microfluidic chip, the effects of different geometry designs and experimental conditions on the transmigration profiles of the cancer cells are studied. Deformations of cancer cells in restricting microchannels during transmigration are also characterized at single-cell level and the critical steps in this process are determined.

3.1 Design of the microfluidic chip

Schematic illustration of the design for the microfluidic cell culture chip is shown in Fig. 3.1. The chip consists of two chambers reservoirs, which are linked by an array of microchannels, each has a height of 5 μm, a length of 100 μm and widths ranging from 4 μm to 12 μm. The microchannels are designed to mimic the small gaps in the extracellular matrix and epithelial syncytium, encountered by the cancer cells during their transmigration. The widths of the microchannels are designed to allow only single cell transmigration. Since the average diameter of cancer cells are 10 to 15 μm, the geometry confinement and the stiffness of PDMS materials will require the deformation of the cells when passing through the narrow channels.

The small dimensions of the microchannels also create high hydrodynamic resistance and greatly limit the flow of the liquid within them, thus diffusion become dominant effect for mass transportation between the two chamber reservoirs. When a high chemical concentration exist in one chamber and a low concentration in the other, the diffusion occurs to buffer the concentration difference between the two chambers, concentration gradients can be established in the mirochannels. When the loaded chemical are signaling molecules stimulating cell migration, the cells cultured on the low concentration side can sense the gradient. The molecular motors inside the cells will be triggered and the cells will subsequently migrate through the microchannels toward high concentrated side. The gradients can be maintained by either initially supplying a large amount of chemicals in the source or frequently replenishing the source and sink through perfusion.

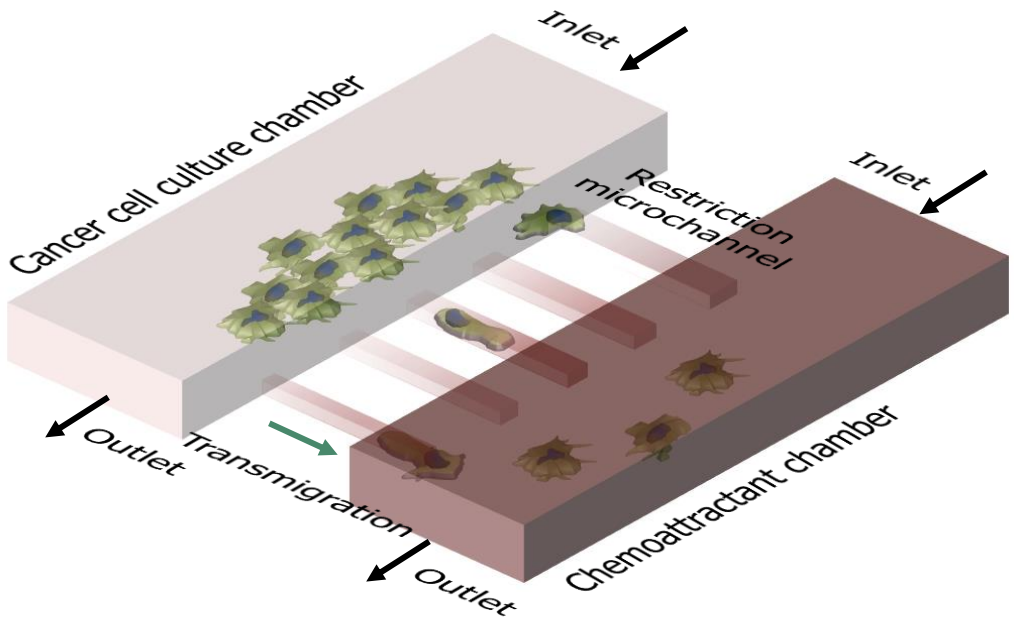


Figure 3.1: Schematic illustration of the microfluidic cancer cell culture chip. Cells are seeded and grown in the cell culture chamber. When a chemoattractant is applied to the chemoattractant chamber, concentration gradients are established along the microchannels and induce the cancer cells to migrate from cancer cell culture chamber to chemoattractant chamber.

3.2 Fabrications and experimental set-up

3.2.1 Fabrication processes

The microfluidic chip is fabricated by using standard soft-lithography techniques [123]. The fabrication process flow is shown in Fig. 3.2. It involves three main steps, namely master patterning, replica moulding and bonding.

1) The master is fabricated on the silicon wafer using lithography techniques. A two-step photolithography method is used in the fabrication process. Firstly, a 5-µm thin layer of SU-8 photoresist (SU-8 10, MicroChem) is spin-coated on a silicon wafer (CEE 200, Brewer Science). After soft baking, it is exposed with the first chrome mask, which patterns the microchannel, and followed by the post exposure bake. After development, a second 100-µm thick layer of SU-8 (SU-8 100, MicroChem) photoresist is spin-coated on top of the first layer and exposed with a second mask to pattern the cell culture chambers and the chemoattractant chamber.

2) PDMS (Sylgard 184, Dow Corning) prepolymer and curing agent mixture (10:1, v/v) are poured over the silicon master, degassed and baked for 2 hours at 75 °C and then peeled off. The inlet and outlet holes are punched manually using a Harris uni-core sampling puncher.

3) The PDMS devices are exposed to air plasma for 15 seconds by using a corona treater (BD-25, Electro-Technic Products) and bonded with glass coverslips.

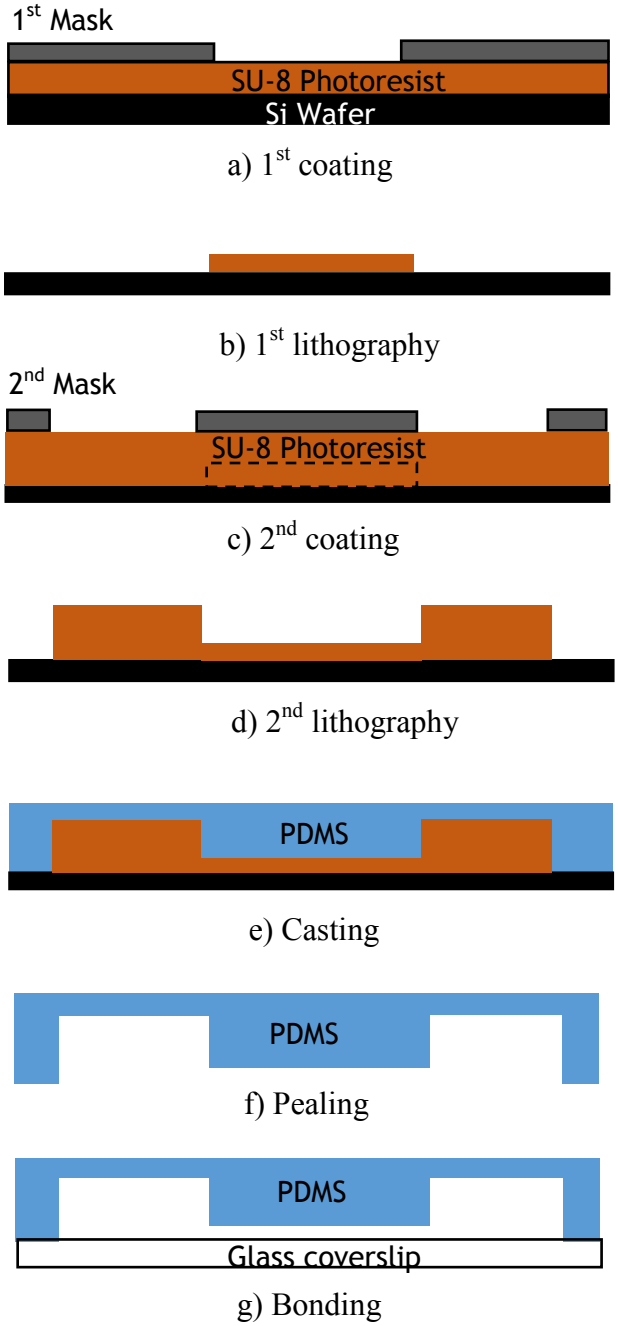


Figure 3.2: Fabrication process of the microfluidic chip.

3.2.2 Cell culture

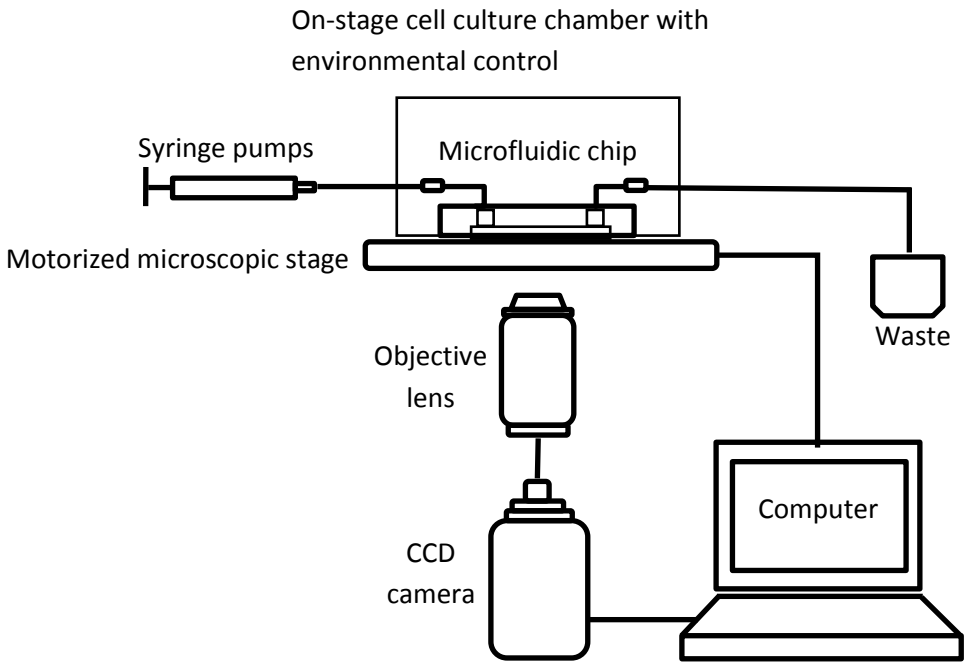
Reagents for cell culture are purchased from Invitrogen. Human breast cancer cells (MDA-MB-231) are maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/ streptomycin (P/S) at 37 ºC and 5% CO_2 environment. Human breast cancer cell line MCF7 is maintained in minimum essential medium (MEM) supplemented with 10% FBS, 1% P/S and 0.01 mg/ml bovine insulin at 37 ºC and 5% CO_2 environment.

3.2.3 Transmigration assay

To enhance cell attachment and migration, 50 µg/ml of fibronectin (Sigma) is injected into the PDMS chip and incubated overnight at 4 ºC. The fibronectin solution is then removed and the chambers are flushed 3 times with 1× phosphate buffer saline (PBS). Adherent cells from the culture flask are detached by treating with trypsin/EDTA (Invitrogen), spun down and resuspended in a growth medium at a density of 1×10^7 cells/ml. To visualize the nucleus of the cells, fluorescent dye Hoechst 33342 (Invitrogen) is added to the cell suspension at a concentration of 1 µg/ml and incubated at 37 ºC for 30 minutes. Cells are then injected into the cell culture chamber and incubated at 37 °C for 2 hours to allow the cells to attach on the substrate and spread. The medium in the cell culture chamber is then replaced with a growth medium without serum. The cells are incubated for 2 hour at 37 °C for serum starvation. Complete growth medium supplemented with different amount of FBS is then added to the chemoattractant chamber.

3.2.4 Experimental set-up

The experimental set-up is shown in Fig. 3.3. It consists of several major components including the microfluidic chip connected with syringe pumps for flow control, a fluorescent microscope equipped with motorized microscopic stage and environmental control chamber, and the computer system connected to the microscope for the collection and analysis of data.



The microscope used in the set-up is Nikon Ti-E microscope equipped with a temperature controlled environmental chamber. Objective lenses used for imaging

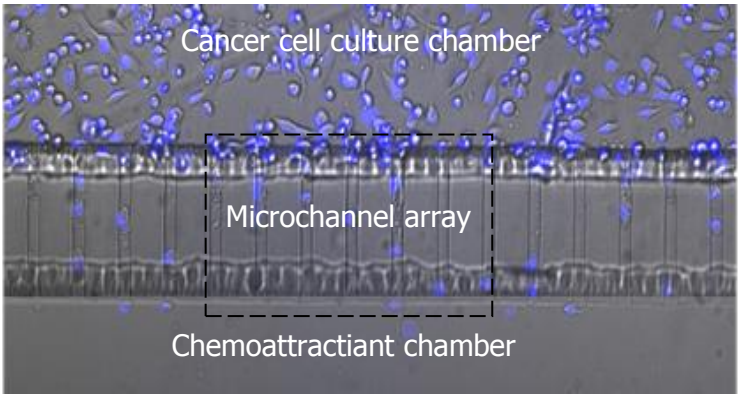
includes Nikon Plan Fluor 10× and 20× dry lenses and a 40× oil lens, with numerical apertures of 0.3, 0.75 and 1.3, respectively. Both digital interference contrast (DIC) and fluorescence images are acquired by using a cooled EM-CCD camera (iXon 885, Andor Technology). Time lapse images of the cells in the microchannels are taken at different time points during the transmigration process. The captured images are analyzed by the NIS-AR Elements software (Nikon).

3.3 Experimental results and discussions

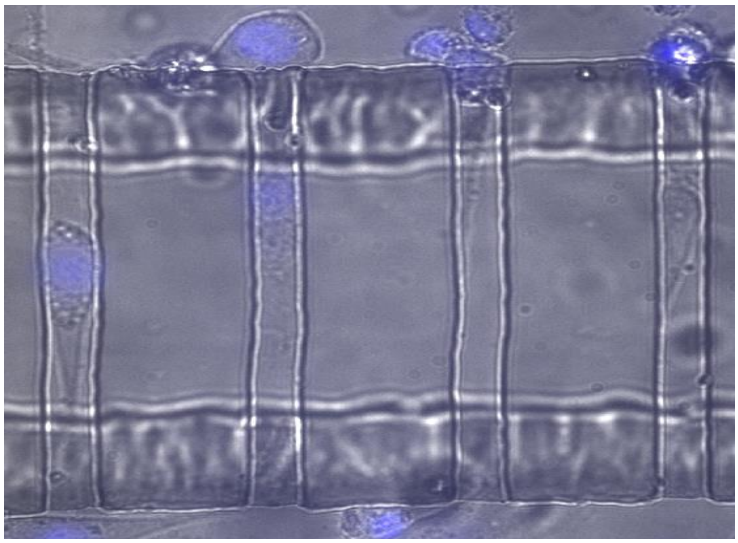
3.3.1 Transmigration of cancer cells

A microfluidic chip with micochannel array mimicking 3D confined environment has been developed. Cancer cells are seeded and cultured in the device under normal cell culture conditions. Figure 3.4a shows breast cancer cells MDA-MB-231 cultured in the microfluidic chip. The cells in the chip have healthy morphologies and are able to migrate from cell culture chamber to chemoattractant chamber.

The cells spread and migrate on the flat surface in front of the channels entrance in a non-directed manner, which is similar to their behavior on flat 2D cell culture surface. Once fetal bovine serum (FBS) is loaded to the chemoattractant chamber after serum starvation, concentration gradients of the chemokines in FBS is established along the microchannels and provide directional cues, the cells in the culture chamber can sense the gradients and attempt to transmigrate through the microchannels to the chemoattractant chamber. This is a general behavior of the cells commonly known as chemotaxis. When transmigrating through the microchannels, the limiting dimensions of channels impose mechanical stress on the cells, depending on the dimensions of the microchannels, the cells deform and reshape themselves to ensure successful transmigration. Cells migrating in 12-μm wide microchannels are not constricted by the PDMS walls, and their morphologies are comparable to cells on flat 2D surface. Cells migrating through microchannels of 8 μm or less in width experience physical confinement by lateral channel walls and deform inwardly to assume an ellipsoid cell shape as shown in Fig. 3.4b.



(a)



(b)

Figure 3.4: MDA-MB-231 breast cancer cells transmigrating in the microfluidic chip. Nuclei of the cells are stained with fluorescent dye Hoechst 33423. DIC and fluorescent images (blue) are over-laid, (a) overview and (b) zoomed-in view.

The microfluidic transmigration chip has several advantages over conventional migration assays. Firstly, the transparent optical qualities of the PDMS microfluidic chip and thin coverglass forming the chamber floor allows good observation of the cells migrating in the microchannels, in combination with microscope automation technique that enables the sequential capture of multiple fields of view, the platform allows the tracking of the movement of large cell populations at single cell resolution. Secondly, the microchannel design is useful in promoting lateral and unidirectional cell movement, which facilitates the analysis of cell velocity and other quantitative parameters of cell migration. Because the dimensions of the microchannels and other internal structures of microfluidic chip can be easily adjusted through the use of customized photomasks, environmental factors affecting cell migration can be studied in highly controlled and repeatable manner.

3.3.2 Nuclear deformation of cancer cells during transmigration

The deformation of cells in the microchannels require the coordination of individual cellular components with distinct physical properties, the microfluidic chip enables the observation of this dynamic process in detail. Figure 3.5 shows the transmigration process of a MDA-MB-231 cell migrating through the microchannels with cross-sections of 8 × 5 μm (W×H), images are taken at different time points during the transmigration process. It occurs in several distinct steps. First, cells come into contact with the entrance of the channel and extend their cytoplasmic portion into it. During this process, the cytoplasm quickly adjusts its shape based on the geometry of the microchannel. It takes approximately 30 minutes for most of the cytoplasm to enter the microchannel, leaving behind the nucleus surrounded by a small amount of cytoplasm. Because of the relatively large nuclear size, the cell is stuck at the entrance. It take a much longer time (approximately 4 hours) for the nucleus to deform and squeeze in. The nucleus deforms from a spherical to an elongated ellipsoidal shape. After the entire cell has entered the microchannel, the cells migrate at a speed approximately 50 μm/hour towards the chemoattractant chamber. Transmigration of cancer cells in narrower channel with 4 μm width also shows similar pattern, during which the cytoplasm can quickly adjusted its shape and enter the channel, leaving the nucleus behind. It takes even longer time for the nucleus to deform and enter the 4 μm channel. The transmigration of the cancer cells are completely inhibited when the channel width is reduced to 2 μm. As shown in Fig. 3.6, the nucleus is stuck at the entrance.

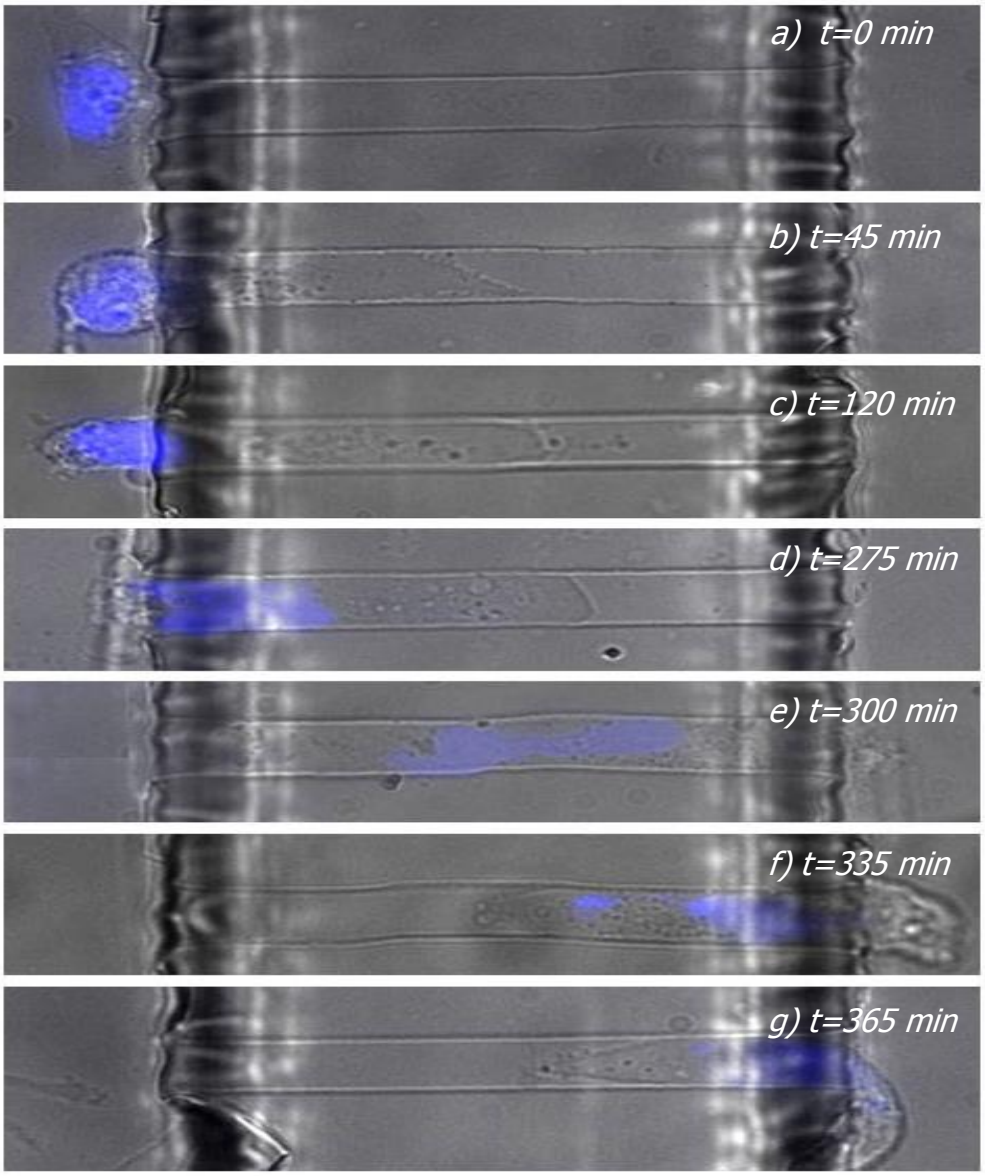


Figure 3.5 Time-lapse images showing the deformation of a MDA-MB-231 cell in the microchannel. Images are taken at different time points from a) t = 0 min to g) t = 365 min during the transmigration process. Nuclei of the cells are stained with fluorescent dye Hoechst 33423. DIC and fluorescent images (blue) are over-laid.

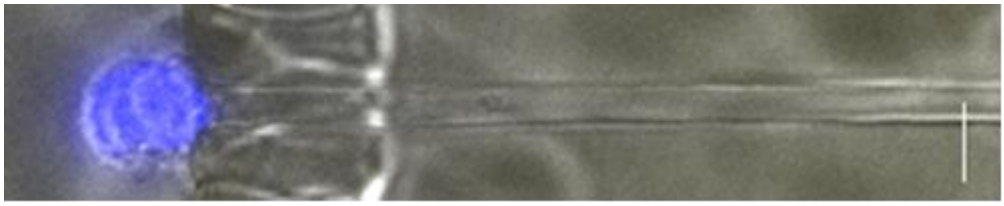


Figure 3.6: Fluorescent photograph shows that a MDA-MB-231 cell is inhibited from entering the 2-μm microchannel.

The results show that the deformation of cell nucleus is the critical and rate-limiting step during the transmigration process, most probably due to its higher stiffness than the rest of the cellular components. The cell nucleus is mainly composed of bio-macromolecules such as DNA, RNA and proteins, the contents vary significantly among different cell types and change dynamically during different stages of cell life cycle, further study on how these molecular factors contribute to the mechanical strength of cell nucleus will help to improve the understanding on cancer cell transmigration and metastasis.

3.3.3 Factors affecting the transmigration profile of cancer cells

To examine the functional consequences of the chemoattractant gradients and the physical restrictions on the transmigration profiles of cancer cells, different concentrations of FBS are loaded into the chemoattractant chamber after serum starvation and the transmigration profile of MDA-MB-231 cells are studied. Figure 3.7 shows the statistics of cells which have migrated through the microchannels under different experimental conditions. When the microchannel width is 12 μm in which the cell deformation during transmigration is insignificant, the amounts of transmigrated cells are well correlated with the concentrations of loaded FBS. When the concentration of FBS is increased stepwise from 0% to 5%, 10% and 20%, percentage of transmigrated cells is also increased correspondingly from 8% to 12%, 27% and 36%. Less amount of cells are able to migrate through the microchannels when the channel width is reduced to 8 μm, but the correlation between the FBS concentration and the percentage of migrated cells are similar. When the channel width is further reduced to 4 μm, in which cell is deformed drastically during transmigration, further reduction in the percentages of transmigrated cells is observed. The effects of increased concentration of FBS on boosting cell transmigration are limited. Maximum percentage of migrated cells (11%) are observed at 10% FBS, further increase of FBS concentration from 10% to 20% does not result in significantly more transmigrated cells.

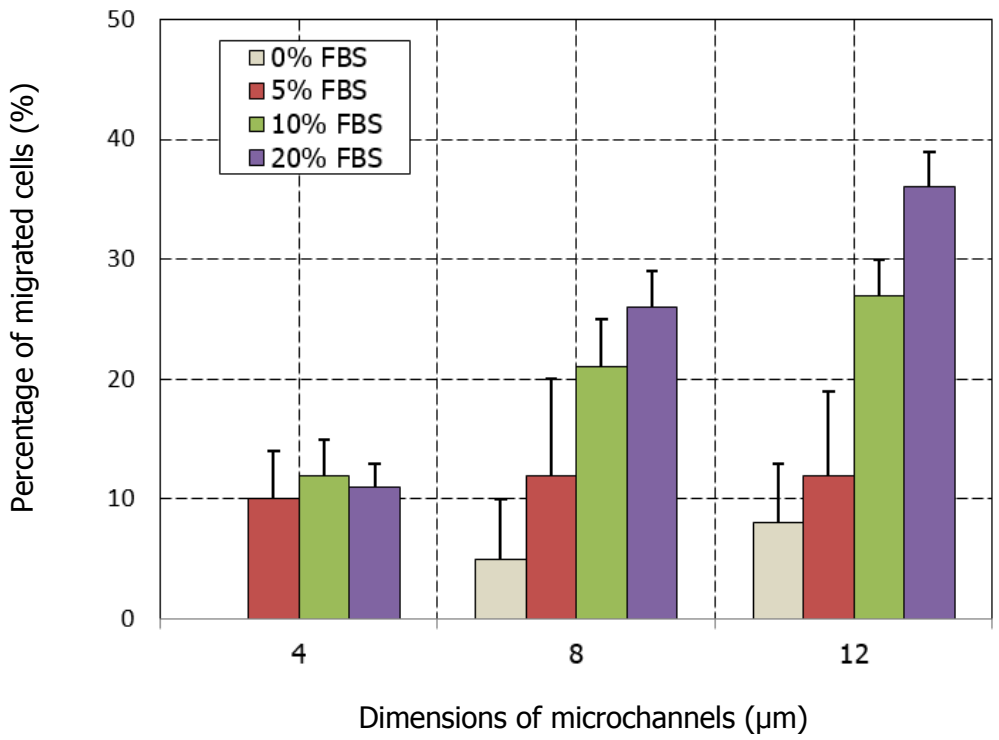


Figure 3.7: Percentage of MDA-MB-231 cells migrate through the microchannels with different concentrations of FBS chemoattraction.

The physical constrains also significantly affect the migration velocities of cells in the microchannels. The migration velocities of MDA-MB-231 cells in different channel width under 20% FBS condition are quantified. As shown in Fig. 3.8, cells migrate at an average of speed of 64 μm/hour inside 12 μm wide microchannels. Cells have slightly lower migration velocity of 54 μm/hr inside microchannels with width of 8 μm. When drastic cellular deformation happens inside the microchannels of 4 μm width, the migration velocity of the cancer cells drops significantly to 21 μm/hr.

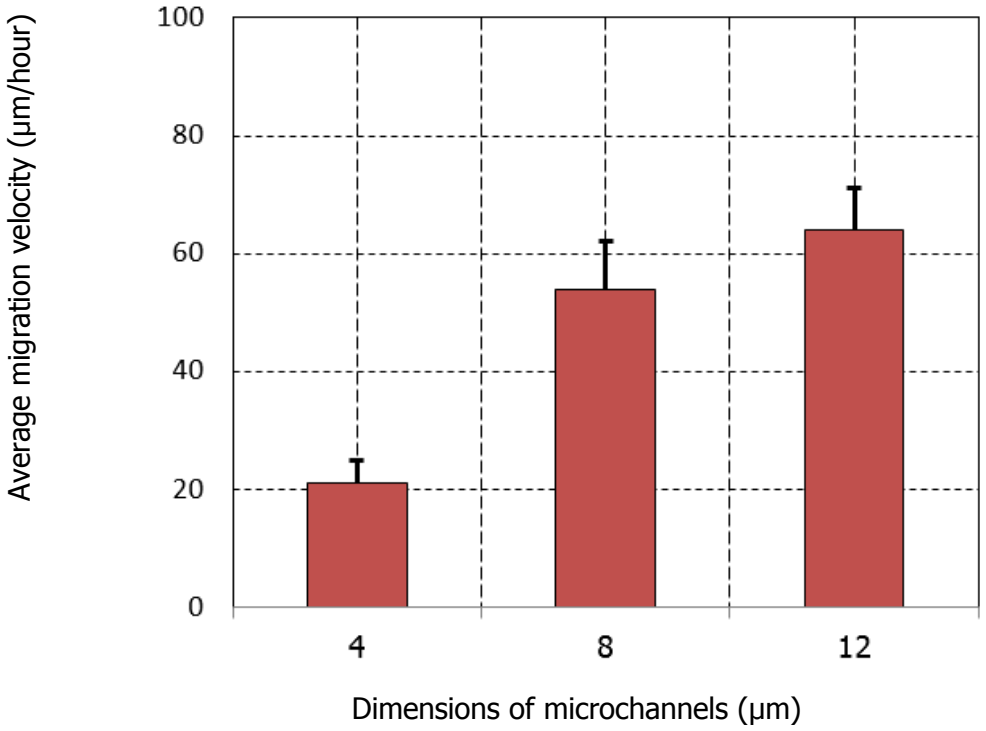


Figure 3.8: The migration velocities of MDA-MBA-231 cells in different widths of microchannels.

Results shows that drastic cellular deformation in the narrow microchannel greatly reduce the chances of cells to migrate through the microchannel arrays and their migration velocities. It cannot be compensated by the increase of chemoattractant which provide directional cues and driven power for migration. Results suggest that the mechanical stress imposed on the cells not only increase the friction between the cells and channel walls, but also affect the intracellular molecular motors responsible for cell migration.

3.3.4 Transmigrations of different cancer cells

Since transmigrations through confined space in extracellular environments are important steps during the metastasis of cancer cells *in vivo*, the transmigration profiles of different types of cancer cells may be related to their metastatic potentials. Using the microfluidic chip, the transmigration profiles of two different breast cancer cell lines MDA-MB-231 and MCF7 are compared. As shown in Fig. 3.9, results show that the highly metastatic MDA-MB-231 cells have higher transmigration capabilities than the lowly metastatic MCF7 cells. Because smaller microchannels require higher extent of cellular deformation, the percentage of transmigrated cells is reduced with the decrease of the microchannel width. In 12-μm wide channels, 36% of the MDA-MB-231 cells migrated across the microchannels in 12 hours after the loading of the chemoattractant. The number drops significantly to 26% in microchannels with 8-μm width and 11% in microchannels with 4-μm width. In each channel dimension, the percentage of transmigrated cells is significantly higher for MDA-MB-231 than for MCF7. The differences are more obvious in narrower microchannels. In 12-μm wide channels, the transmigration rates are 36% *vs* 28%. However, the transmigration rate of MDA-MB-231 cells (11%) is almost 3-fold higher than that of MCF7 (4%) in 4μm wide channels.

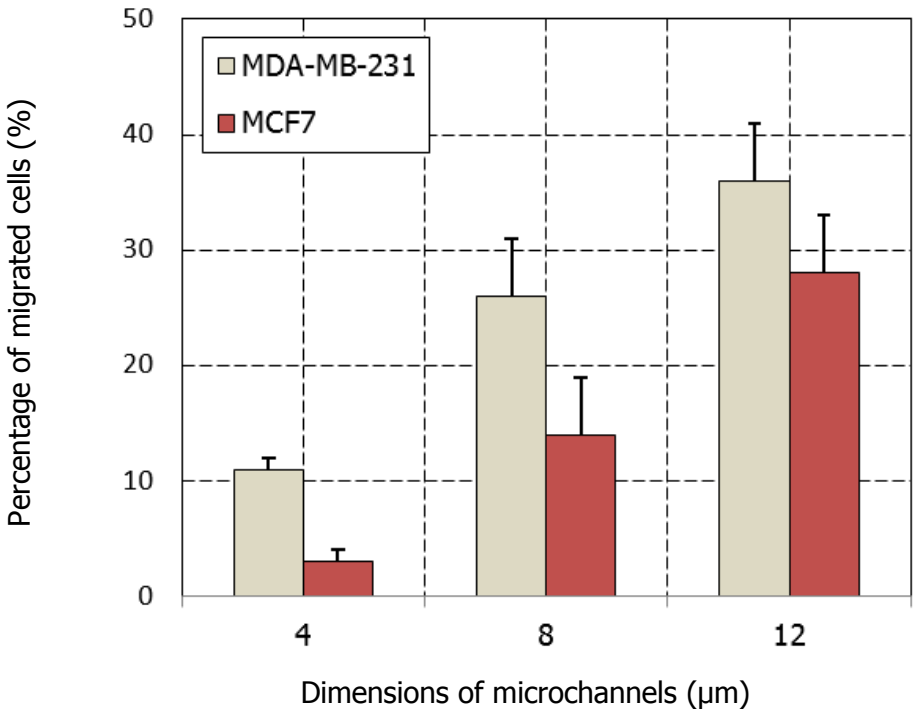


Figure 3.9: Transmigration profiles of MDA-MB-231 cells and MCF7 cells in microchannels with different width.

The results in earlier sections show that the deformation of cancer cells, especially their nucleus are important for cancer cell transmigration in microchannels. Although a direct comparison on the mechanical properties of these two cell types has not yet been performed, the results suggest that MDA-MD-231 cells have a higher deformation capability than MCF7 cells, which may facilitate their transmigration during metastasis.

The microfluidic cell culture chip may have potential applications in cancer diagnosis. By accessing transmigration capabilities of different types of cancer cells, their metastatic potential, which is an important indicator for the stage information of cancer disease, can be evaluated on the microfluidic chips.

3.4 Summary

In this chapter, a microfluidic cell culture chip for the study of cancer cell transmigration and metastasis is presented. The design of the microfluidic chip is demonstrated in section §3.1, it uses microchannels with dimension from 4-12 µm to mimic the tissue capillaries which cancer cells encounter during their metastasis *in vivo*. The two-step soft-lithography processes for the fabrication of microfluidic chip and the experimental methods are shown in section §3.2. The experimental results are shown in section §3.3, the influences of geometric confinement on cell transmigration are studied using microchannels of varying dimensions, results show that the deformation of the stiff cell nucleus is the most time-consuming step during the transmigration process. Drastic cell deformation limits the number of cells transmigrating through the microchannels, when the channel width is reduced from 12 µm to 4 µm, the percentage of transmigrated cells drops from 36% to 11%. The transmigration capabilities of two types of cancer cells MDA-MB-231 and MCF7 are compared, results show that highly metastatic MDA-MB-231 cells can migrate through the microchannels more efficiently than lowly metastatic MCF 7 cells.

In summary, the microfluidic cancer cell culture chip presented in this chapter will be useful tool for the study of cancer cell metastasis and the diagnosis of cancer disease.

CHAPTER 4

NUCLEAR DEFORMATION AND CHROMATIN CONDENSATION OF TRANSMIGRATED CANCER CELLS

The deformation of cancer cells, especially their nucleuses are critical for their transmigration in the confined geometries of the microchannels. In this chapter, the transmigrated cancer cells are further analyzed to explore whether the mechanical stress can change the chemical or biological properties of the cancer cells.

The chemical properties of the transmigrated cancer nucleus, including the chromatin condensation and histone modification status, are firstly analyzed in section §4.1 and §4.2. The chromatins in cell nucleus are stained with fluorescent dye DAPI and their condensation status is analyzed in real-time during the transmigration of the cancer cells. The nuclear histone proteins are stained with different fluorescent-labelled antibodies which are able to recognize specific modifications on the histone proteins.

To study whether the changes in the chemical properties of the transmigrated cancer cell can be inherited after cell division, the transmigrated cancer cells are isolated from the microfluidic chip and sub-cultured in other cell culture devices in section §4.3. Different biochemical methods including immunofluorescence assay and western blot are used to analyze the daughter cells.

The biological property on the transmigration capabilities of different cancer cells are then investigated in section §4.4. Transmigration assay are performed on the cancer cells harvested from both the microfluidic chip and other cell culture devices.

In the last section, pharmacological approaches to prevent cancer transmigration are explored. Specific drugs with capabilities of blocking chromatin condensation and histone modifications are applied to the cancer cells and the cell transmigration profiles in the microchannel array are studied.

The methods used for the analysis of transmigrated cancer cells are summarized in Tab. 4.1.

Cell culture devices	Biochemical method used to analyze the transmigrated cancer cells
Microfluidic chip	DAPI staining Immunofluorescence assay
Cell culture dish	Immunofluorescence assay Transmigration assay
Cell culture flask	Immunofluorescence assay Western-blot assay Transmigration assay

Table 4.1 Different biochemical method used to analyze the transmigrated cancer cells

4.1 Chromatin condensation in the transmigrated cancer cells

4.1.1 Experimental methods

The chromatin condensation of the transmigrated cancer cells is monitored by staining the nucleus of the cancer cell with fluorescent dye. The cancer cell and fluorescent dye used here are MDA-MB-231 cells and 4',6-diamidino-2-phenylindole (DAPI), correspondingly. DAPI binds strongly to DNA molecules on the chromatins of the cancer cell and the staining intensity is correlated to the condensation status of nucleus chromatin.

The staining is carried out by first loading MDA-MB-231 cells to the cancer cell culture chamber. After the cells have been loaded in to the microfluidic chip and attached to glass substrate, the DAPI reagent (Sigma) is then added to the cell culture chamber at a final concentration of 1 μg/ml. Next, the cells are incubated at 37 ºC for 30 minutes and finally transmigration assay is performed using protocols described in section §3.2.3. The intensity of DAPI staining during the transmigration is monitored using fluorescent microscopy. Time lapse images of the transmigration of DAPI-stained cells in the microchannels are captured and analyzed using NIS-AR Elements software.

4.1.2 Experimental results and discussions

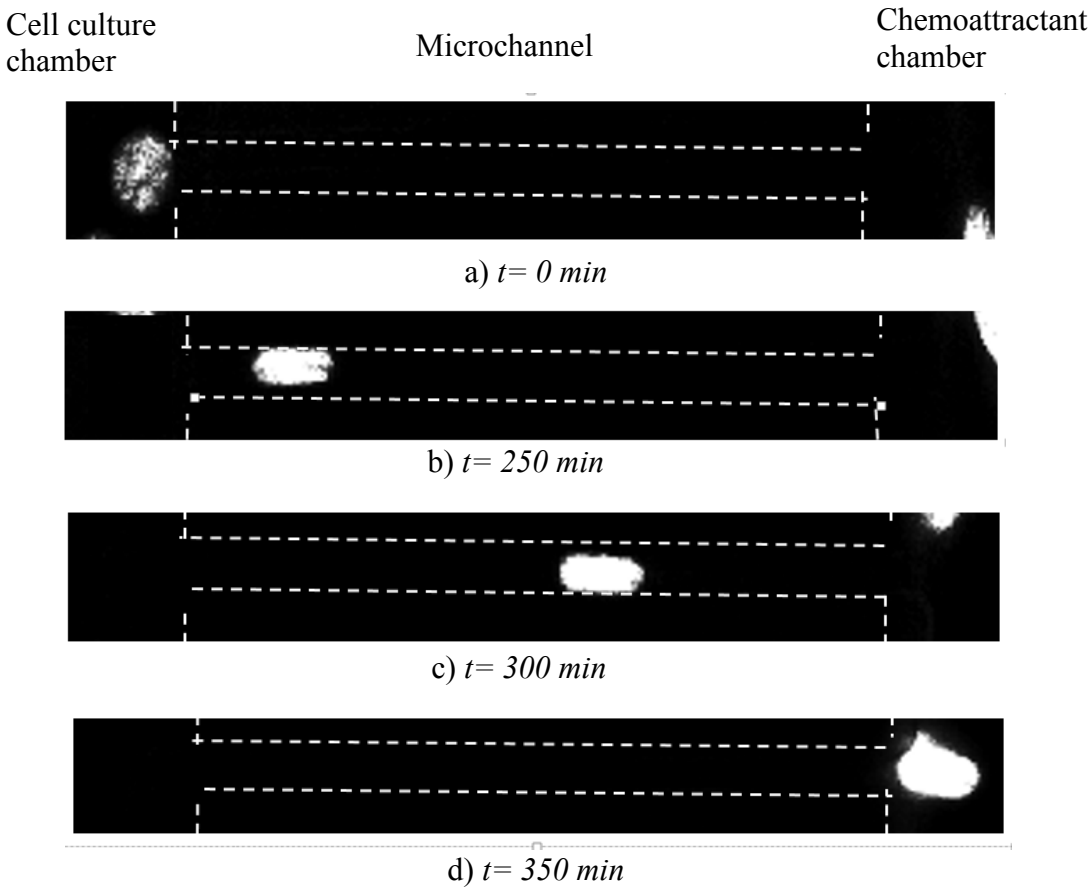
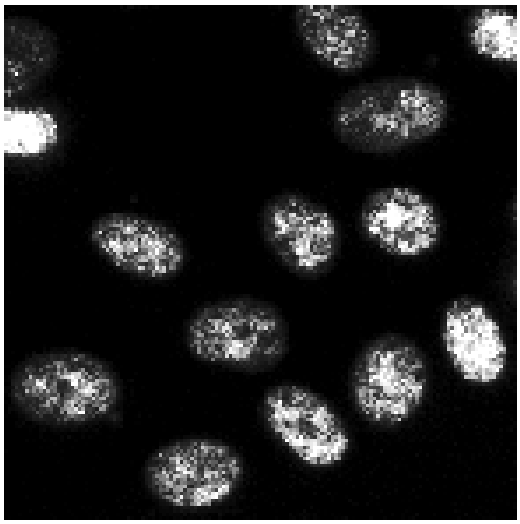
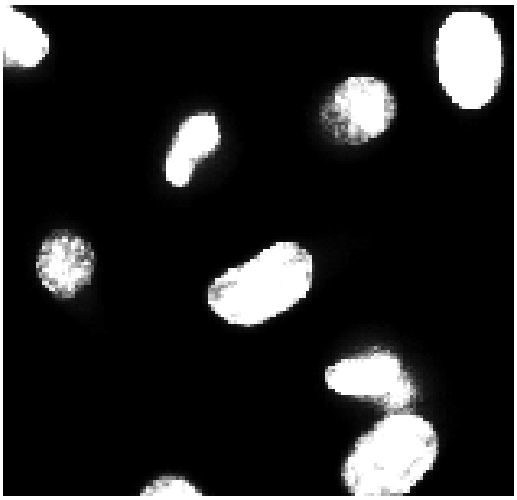


Figure 4.1: Fluorescent images showing the migration of a MDA-MB-231 cell through a microchannel with cross-section of 4 ×5 μm. The nucleus of the cell is stained with fluorescent dye DAPI. Increase of DAPI staining intensity can be observed when cancer cell are migrating in the microchannels, suggesting that chromatin condensation happens during cancer cell transmigration.



(a) Cells which stay in the cancer cell culture chamber



(b) Cells which have transmigrated into the chemoattractant chamber

Figure 4.2: Fluorescent images of (a) cells which stays in the cancer cell culture chamber (b) cells which have transmigrated into the chemoattractant chamber. The transmigrated cells have significantly higher DAPI staining intensities, suggesting that they have more condensed chromatins than non-transmigrated cells.

Changes in the DAPI staining intensity during the transmigration of one MDA-MB-231 cell are shown in Fig. 4.2. The intensity is relatively stable before the cell enters the microchannel, while starts to change shortly after the cell squeezes itself into the microchannel. The intensity keeps elevating when the cell is migrating along the microchannel to the chemoattraction chamber. Maximum intensity is observed when the cell arrives at the end of the microchannel. After the cell leaves the microchannel and migrates into the chemoattractant chamber, the staining intensity of the cell remains relatively constant.

The overall staining intensities of the cells left in the cancer cell culture chamber and of those transmigrating into the chemoattraction chamber are compared in Fig. 4.3. Significantly higher DAPI-staining intensity is observed from the transmigrated cells, suggesting that they have more condensed chromatins than non-transmigrated cells.

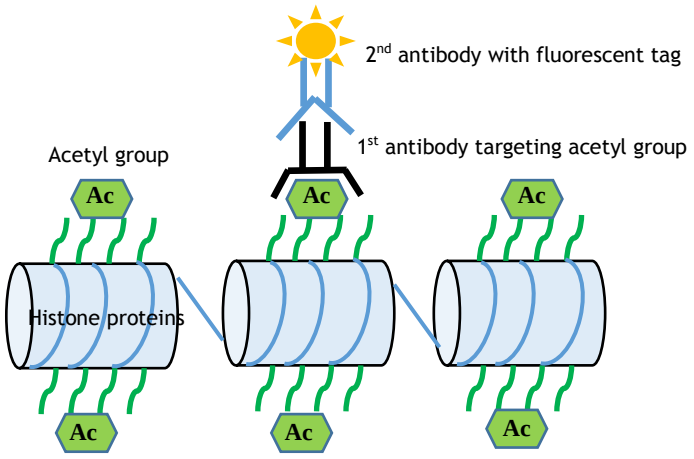
In summary, the staining of transmigrated cancer cell with fluorescent dye DAPI shows that the transmigration of cancer cells induces chromatin condensation in their nucleus, which may be caused by the mechanical stresses imposed on the cells by the constricting geometries of the microchannels.

4.2 Histone modifications in the transmigrated cancer cells

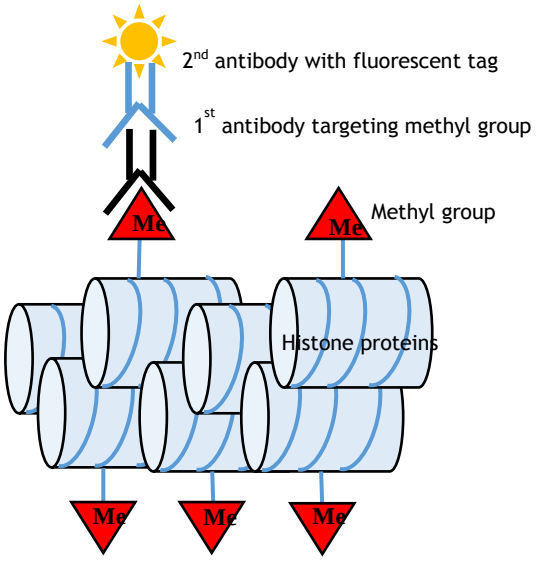
4.2.1 Experimental methods

Histone proteins are major components of nuclear chromatin, changes of chromatin condensation status are usually associated with the biochemical modifications on the histone proteins. There are two major types of modified histone proteins exist in cell nucleus: 1) methylated histones which are usually found in condensed chromatin and 2) acetylated histones which are usually found in loose chromatin [124, 125]. Modified histone proteins can be detected by immunofluorescence assay with fluorescent antibodies, the working principles of chromatin immunofluorescence staining are illustrated in Fig. 4.3.

To detect the modified histone proteins in transmigrated cancer cells, the microfluidic chip is dislodged from the glass coverslip at the end of the transmigration assay. The coverslip is soaked in 4% (w/v) para-formaldehyde (Sigma) solution for 30 minutes at room temperature to fix the cells. Cells are firmly attach to the glass substrate after fixation. After being washed three times with PBS, the cells are incubated with 0.5% (v/v) TritonX-100 (Sigma) in PBS for 1 hour to permeablize the cell and nucleus membrane. Antibodies against specific histone proteins (Abcam, Cambridge, USA) are diluted 1:500 in blocking buffer (0.5% TritonX-100 and 2% bovine serum albumin in PBS). The diluted antibody solutions are added to the cells and incubated for 2 hours at 37°C. After washing 3 times with PBS, cell are incubated for another 1 hour at room temperature with alexa-fluor tagged secondary antibody (Abcam) diluted 1:1000 in blocking buffer to visualize the stained histone protein.



(a) Detection of acetylated histones in loose chromatins

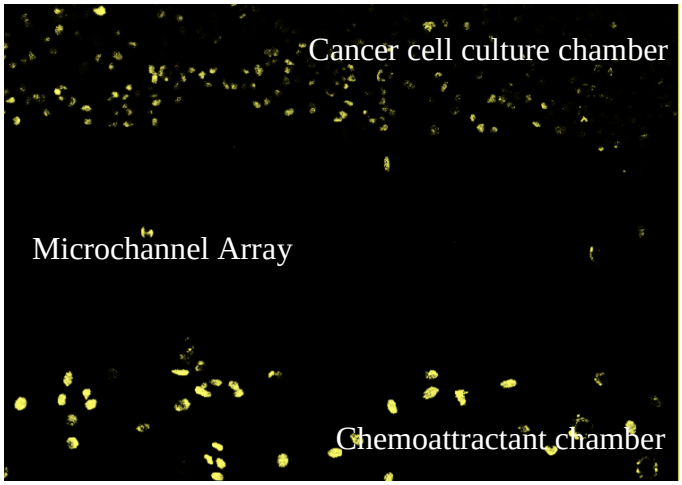


(b) Detection of methylated histones in condensed chromatins

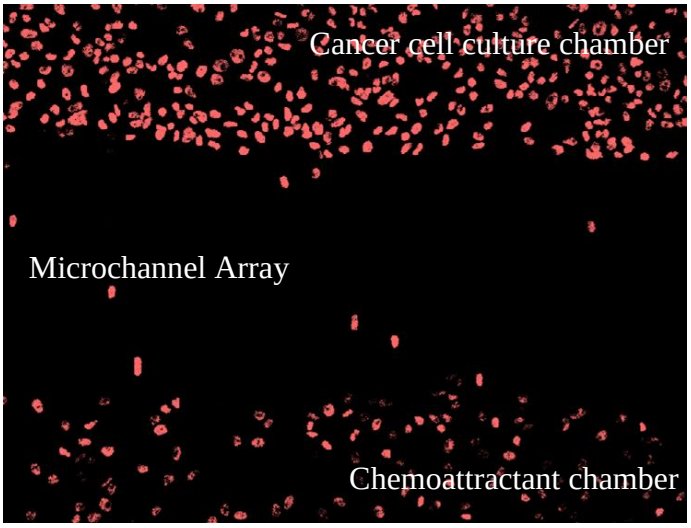
Figure 4.3: Schematic of histone immunofluorescence staining. (a) Acetylated histone proteins in loose chromatin and (b) methylated histones in condensed chromatins are detected by different combinations of fluorescent-label antibodies.

4.2.2 Experimental results and discussions

Three different antibodies are used in the experiment, two of them recognize methylated histones mono-methyl Lysine20 in H4 histone (H4K20Me1) and tri-methyl Lysine9 in H3 histone (H3K9Me3) which are found in condensed chromatin, and another one recognizes acetylated histone Lysine9 in H3 histone (H3K9ac) found in loose chromatin. Representative fluorescent images are shown in Fig. 4.4. As revealed by the immunofluorescent staining, the transmigrated cells have significantly higher staining intensities than the non-transmigrated cells when the cells are stained with antibodies recognizing methylated histones. On the other hand, the staining intensity on the transmigrated cells will drop dramatically when the cells are stained with antibodies recognizing acetylated histone proteins.



(a)



(b)

Figure 4.4: Fluorescent images of histone proteins from MDA-MB-231 cells migrating in the microchannels with cross-section of 8 × 5 μm. (a) Cells are stained with antibodies recognizing methylated histone H4K20Me1. (b) Cells are stained with antibodies recognizing acetylated histone H3K9Ac.

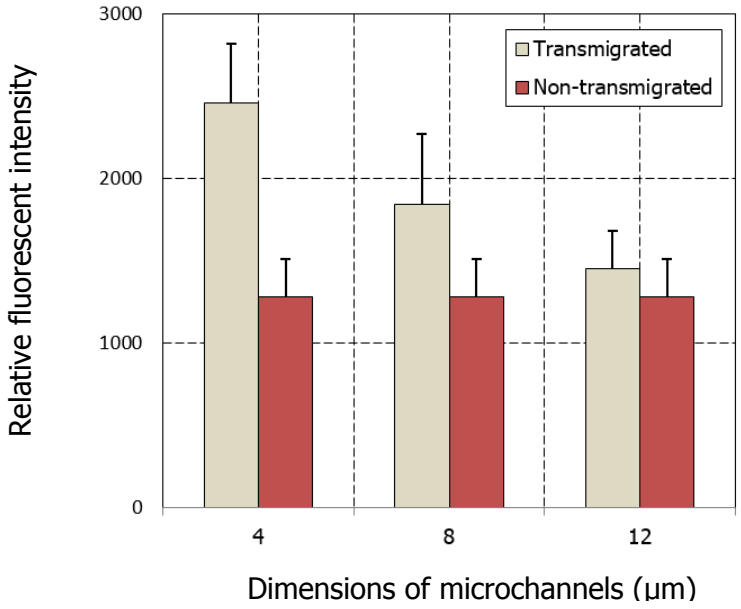


Figure 4.5: Statistical results of immunofluorescent staining intensities with antibodies recognizing methylated histone H4K20Me1.

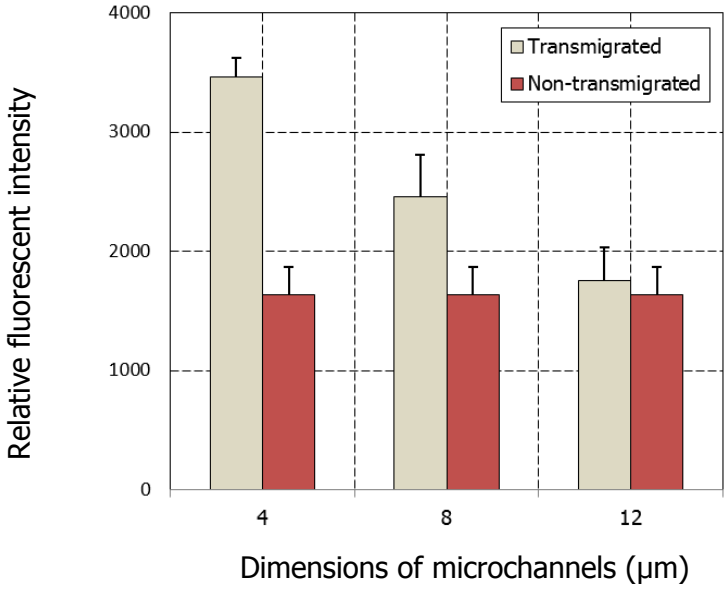


Figure 4.6: Statistical results of immunofluorescent staining intensities with antibodies recognizing methylated histone H3K9Me3.

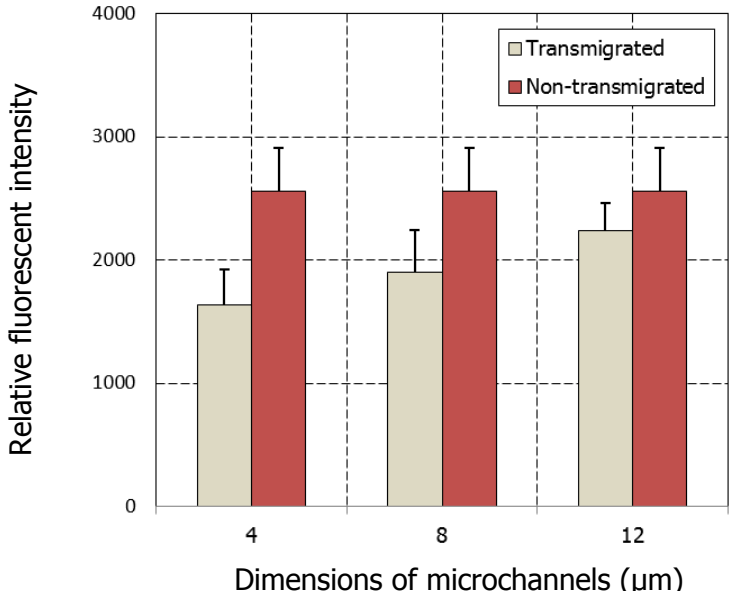


Figure 4.7: Statistical results of immunofluorescent staining intensities with antibodies recognizing acetylated histone H3K9Ac.

Figure 4.5 shows the staining intensity of cancer cells which have transmigrated through microchannels with different widths. The cells are stained with antibodies which recognizes methylated histone H4K20me1. An average fluorescent intensity of 1376 is observed for cells migrating through 12-μm wide channels, which is close to the intensity 1288 of non-transmigrated cells. When the channel width is reduced 8 μm, the intensity significantly increases to 1846. When the channel width is further narrowed to 4 μm, in which drastic cellular deformation happens during transmigration, the average staining intensity increases almost 1-fold to 2460. The staining intensity of another methylated histone H3K9Me3 is also studied. As shown in Fig. 4.6, the average intensities are

increased from 1640 of the non-transmigrated cells to 3460, 2477 and 1754, after the cells migrate through 4-μm, 8-μm and 12-μm wide microchannels, respectively.

On the contrary, immunofluorescent staining with antibodies recognizing acetylated histone H3K9Ac shows different trends. As shown in Fig. 4.7, the staining intensities are decreased for cells which have transmigrated through the microchannels compared to the non-transmigrated ones. The decrease is also correlated to the dimension of the microchannels: the average intensities are decreased from 2560 of the non-transmigrated cells to 1635, 1896 and 2240, after the cells migrate through 4 μm, 8 μm and 12 μm wide microchannels, respectively.

In summary, the histone proteins of the cancer cells are biochemically modified after the cells transmigrate through the microchannels. As results, more methylated histone and less acetylated histones in the nucleus of transmigrated cancer cells are detected. Since the methylated histone proteins are commonly found in condensed chromatins, result suggests that chromatin condensation happened during cancer cell transmigration, which are consistent with the results presented in section §4.1.

Histone modifications and chromatin condensations may have further implications on cell behavior since they are closely associated with diverse biological processes such as gene regulation, DNA repair and cell division. Further studies at cellular and molecular biology level might lead to the discovery of other important consequences of the changes in cancer cells' chemical properties.

4.3 Chromatin condensation and histone modification status after cell division

It has been reported that the condensed chromatin and some of the biochemically modified histone proteins are stable biomarker in cells which can be kept after cell division [125]. In this section, experiments are carried out to explore whether the chromatin condensation and histone modification status induced by the transmigration of cancer cells in microchannels can be inherited by their daughter cells after cell divisions.

4.3.1 Experimental methods

Sub-culture of transmigrated cancer cells

The transmigrated cancer cells (MDA-MB-231) are first isolated from the chemoattractant chambers in microfluidic chips by treating them with trypsin-EDTA reagent (Sigma) for 2 minutes at 37 °C. The detached cells are seeded in 35-mm diameter cell culture dish and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/ streptomycin (P/S) at 37 ºC and 5% CO_2 environment. After the cells grow confluent, they are detached from the cell culture by trypsin-EDTA treatment. Some of the cells are used for analysis and the rest further propagate into T-25 tissue culture flask with bigger area for cell growth. When the cells grow confluent in cell flask, they are detached from the flask and used for further analysis.

Immunofluorescence analysis

The cells harvested from cell cultured dishes and flasks are seeded on glass coverslips and the immunofluorescent assay are carried out using protocols described in section §4.2.1.

Western-Blot Analysis

Cells harvested from tissue culture flask are dissolved in lysis buffer (50 mM Tris HCl, pH 8.0, 150mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1:100 dilution of protease inhibitor mixture (Sigma-Aldrich)), and sonicated. To assure equal protein loading, the concentrations of the lysates are determined with the DC Protein Assay kit according to the microplate protocol (Bio-Rad). Samples consisting of 25 µg of lysate protein are separated using SDS-PAGE on 10% Tris-glycine gels (Invitrogen). Proteins are transferred to polyvinylidene difluoride (PVDF) membranes (Invitrogen) and immunoblotted as follows. The PVDF membranes are blocked with 5% nonfat milk in PBS for 1 hour at room temperature. The blots are then incubated with the histone marker antibody diluted 1:100 in the blocking solution overnight at 4 °C and then washed 3 times for 10 minutes each with PBS plus 0.1% Tween-20 at room temperature. The membranes are incubated with goat anti-mouse horseradish peroxidase (Santa Cruz Biotechnology) diluted 1:1000 in blocking solution for 1 hour. The membranes are then washed 4 times for 15 minutes each with PBS plus 0.1% Tween-20, exposed with

SuperSignal West Pico chemiluminescent horseradish peroxidase substrate (Pierce), and developed on x-ray film according to the instructions.

4.3.2 Experimental results and discussions

Immunofluorescent staining results

Cancer cells harvested from cell culture dish and tissue culture flask are analyzed by immunofluorescence assay using antibodies recognizing different histone proteins. As shown in Fig. 4.8, the statistical results show that the staining intensities of the daughter cells harvested from the cell culture dish are higher in methylated histones and lower in acetylated histones than that of non-transmigrated cells on the cancer cell culture chamber of the microfluidic chip. This shows same trend as the transmigrated cells in the chemoattractant chamber of the microfluidic chip, which demonstrate the heredity of the daughter cells from the cell culture dish. However, the results also show that the histone modification status cannot last infinitely during repeated cycles of cell divisions. When the cells are further sub-cultured from cell culture dish into tissue culture flask, the staining intensities of harvested cells from the flasks recovers to levels comparable to those of non-transmigrated cells.

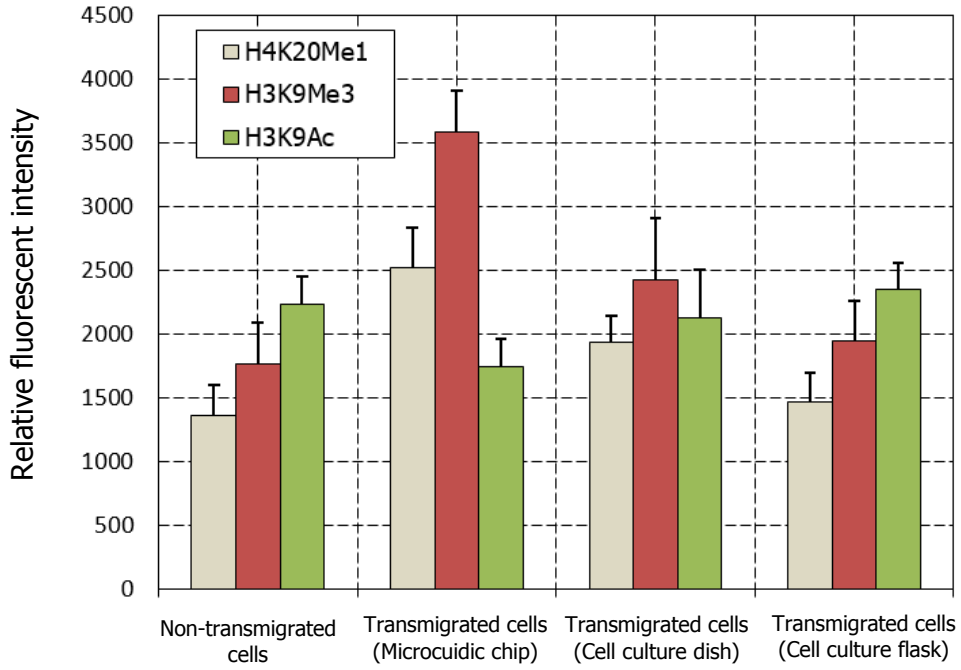
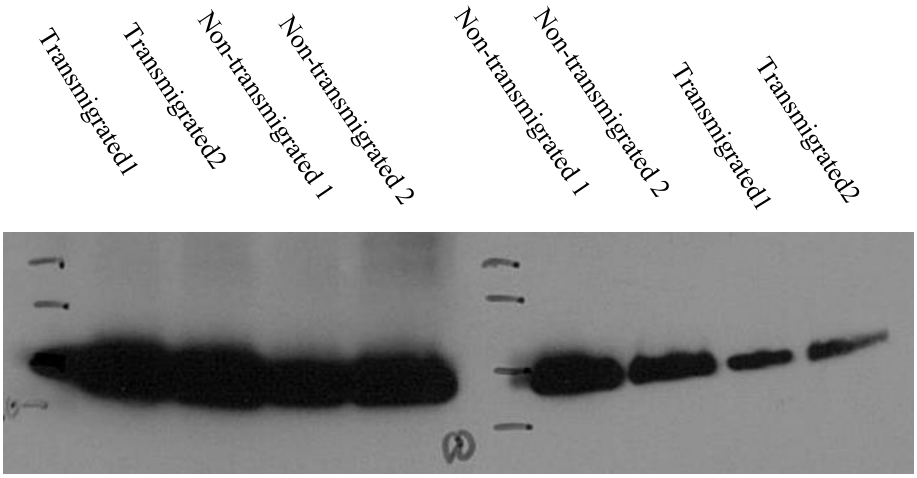


Figure 4.8: Statistical results of immunofluorescence staining intensities of sub-cultured cancer cells. Non-transmigrated refer to those cells remain in the cancer cell culture chamber in the microfluidic chips, transmigrated cells (microfuidic chip) refer to those cells which have migrated though the microchannels into the chemoattractant chamber in the microfluidic chip, transmigrated cells (cell culture dish) refers to the sub-cultured cells harvested from cell culture dish, transmigrated cells (cell culture flask) refers to the sub-cultured cells harvested from cell culture flask.

Western-blot results



Antibodies detecting
H3K9Me3 methylated histones

Antibodies detecting
H3K9Ac acetylated histones

Figure 4.9: Western-blot analysis of MDA-MB-231 cells harvested from tissue culture flask. Proteins in cells are separated vertically based on their molecular weights and detected by specific antibodies recognizing modified histone proteins. Non-transmigrated cells refer to the original cells which have not been used in the microfluidic chips. Transmigrated cells refer to those sub-cultured cells harvested from tissue culture flask.

Figure 4.9 shows the results of western-blot assay. Different proteins in the cell lysates are separated vertically on the PVDF membranes according to their molecular weights and modified histone proteins are detected by specific antibodies. Thicknesses of the bands are correlated to the amount of the modified histone proteins in cell lysate. The results are consistent with the immunofluorescent staining analysis. Cancer cells harvested from tissue culture flask shows slightly higher amount of methylated histone proteins and lower acetylated histone amounts than non-transmigrated cells, but the differences are not significant.

In summary, the results of immunofluorecent staining and western blot analysis show that histone modification and chromatin condensation induced by the transmigration of cancer cells in microchannels can be inherited and kept in cells for certain period, it will gradually recover to normal level after repeated cycles of cell division. Since the cell are grown on flat surface without deformation during sub-culture, which is different with the conditions in the microfluidic chips, results suggest that the mechanical stress imposed on cancer cells by the restricting microchannels are indispensable for the induction and maintenance of chromatin condensation and histone modification status in cells.

4.4 Transmigration capabilities of sub-cultured cancer cells

Chromatin and histone proteins occupy substantial volume of the cell nucleus. The condensation status of chromatins is a major determining factor for nuclear mechanical properties. In this section, experiments are carried out to explore whether chromatin condensation and histone modification status inherited by the sub-cultured cancer cells will result in the change of their transmigration capabilities.

4.4.1 Experimental methods

The cells harvested from cell cultured dish and flasks are reloaded in to the cancer cell culture chamber in the microfluidic chips at a density of 1×10^7 cells/ml. The microfluidic chips are incubated at 37 °C for 2 hours to allow the cells to attach on the substrate and spread. The medium in the microfluidic chip is then replaced with a growth medium without serum. The cells are incubated for 2 hour at 37 °C for serum starvation. Complete growth medium supplemented with 20% FBS is then added to the chemoattractant chamber to induce cell migration.

4.4.2 Experimental results and discussions

The transmigration profiles of the sub-cultured cancer cells are compared with the cells which have not been used in the microfluidic chip, referred as “non-transmigrated cells” in Fig. 4.10. As shown by the statistical results, the cells harvested from cell culture dish, which inherit the chromatin condensation and histone modification status from their mother cells, have higher transmigration capabilities than the non-

transmigrated cells for all three different microchannel widths. The relatively high transmigration capability is especially significant in the case of 4-μm wide channels where drastic nuclear deformation is required during transmigration: the percentage of transmigrated cells harvested from cell culture dish (18%) is much higher than that of non-transmigrated cells (11%).

On the other hand, for different channel widths of 4 μm, 8 μm and 12 μm, the transmigrated cell percentage are 9%, 28% and 37% for the cells harvested from tissue culture flask, and 11%, 26% and 36% for the non-transmigrated cells. Therefore, the cells harvested from tissue culture flasks exhibit similar transmigration capabilities to the non-transmigrated cells.

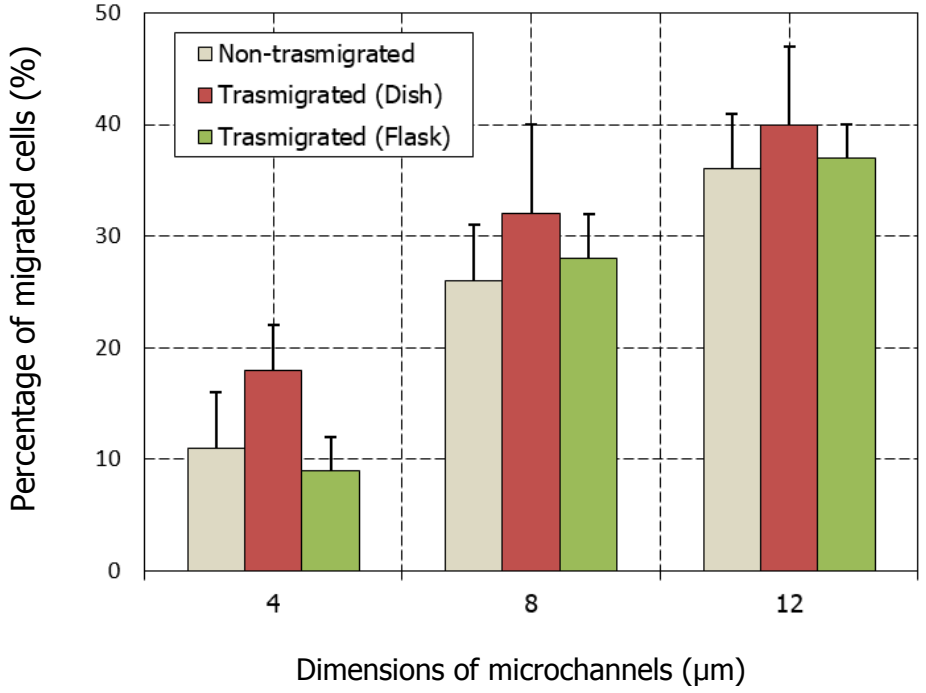


Figure 4.10: Statistical results of transmigration profiles of sub-cultured cancer cells. Non-transmigrated cells refer to the original cells which have not been in the microfluidic chip. Transmigrated (dish) refer to those sub-cultured cells harvested from cell culture dish. Transmigrated (flask) refer to those sub-cultured cells harvested from tissue culture flask.

In summary, the chromatin condensation and histone modification can cause the increase in cancer cells' transmigration capabilities, which is most probably because the chromatin condensation facilitates nuclear reshaping and squeezing and makes it easier for cells to go through the confined space during transmigration.

4.5 Inhibition of cancer cell transmigration by histone-modulating drugs

The experimental results shows that transmigration of cancer cells in the microchannels can induce chromatin condensation and histone modification in their nucleus. In this section, experiments are carried out to study whether blocking histone modification through pharmacological approaches can inhibit the transmigration of cancer cells in the microchannels.

4.5.1 Experimental methods

Drug treatment

5'-Deoxy-5'-methylthioadenosine (MTA) is a general protein methyltransferase inhibitor, which blocks the transfer of methyl group to specific lysine residues on histones and consequently inhibits the formation of methylated histone proteins [126]. To treat the cancer cells with MTA drug, the stock solution of MTA (Sigma) is prepared by dissolving the MTA powder into dimethyl sulfoxide (DMSO) at a concentration of 300 mM. During experiments, MTA stock solution is diluted with the culture medium to a final concentration of 3-300 μM for the treatment of cancer cells.

Immunofluorescent assay

MDA-MB-231 cells are seeded and grown on glass coverslip, cells are treat with 300 μM MTA for 12 hours and then fixed and immunostained with antibodies

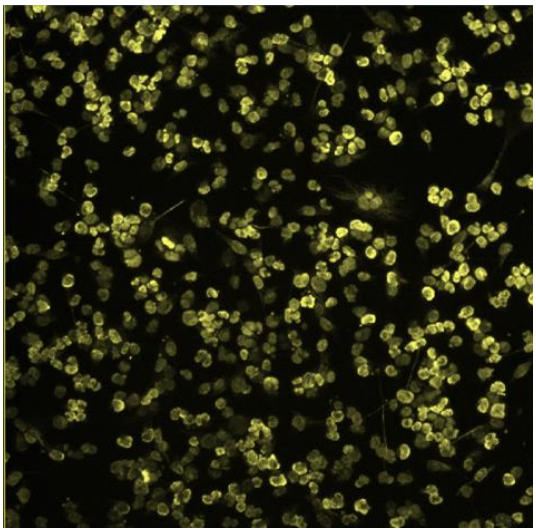
recognizing methylated histone H4K20me1 using the protocols described in section §4.2.1.

Transmigration assay

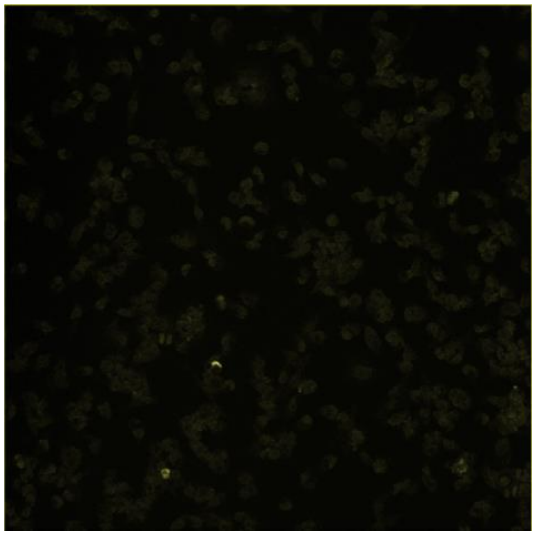
MDA-MB-231 cells are loaded into the cancer cell culture chamber in the microfluidic chips at a density of 1×10^7 cells/mL. The microfluidic chips are incubated at 37 °C for 2 hours to allow the cells to attach on the substrate and spread. The medium in the microfluidic chip is then replaced with a growth medium without serum. The cells are incubated for 2 hour at 37 °C for serum starvation. Complete growth medium supplemented with 20% FBS is then added to the chemoattractant chamber to induce cell migration. Meanwhile MTA stock solution is added to cancer cell culture chamber to a final concentration of 3-300 μM for the treatment of cancer cells.

4.5.2 Experimental results and discussions

The effect of MTA on blocking cellular histone modification is first verified by immunofluorescent staining. After being treated with 300 μM MTA for 12 hours, the MDA-MB-231 cells are stained with fluorescent antibodies recognizing methylated histone H4K20Me1. The fluorescent images shown in Fig. 4.11 indicate that MTA treatment significantly reduces the amount of methylated histones formed in cell nucleus, the cells treated with MTA drug have significantly lower staining intensity than those without drug treatment.



(a) MDA-MB-231 cells not treated with MTA drug

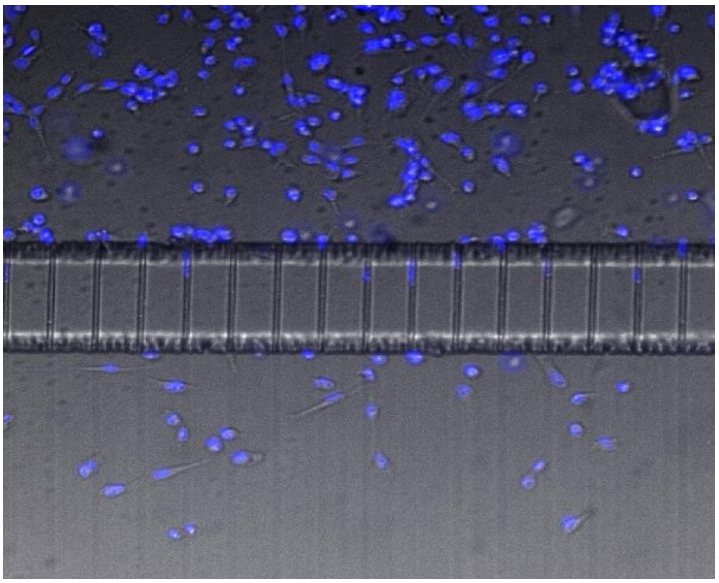


(b) MDA-MB-231 cells treated with MTA drug

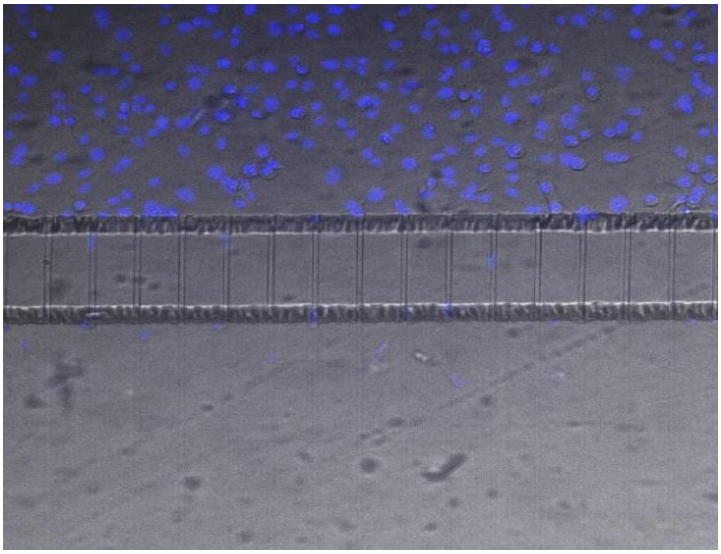
Figure 4.11: Fluorescent images of MDA-MB-231 cells (a) not treated with MTA drug (b) treated with MTA drug, immnostained with antibodies recognizing methylated histone H4K20Me1.

The effect of MTA on the transmigration of MDA-MB-231 cells in the microchannel is shown in Fig. 4.12. It is shown that MTA drug inhibits the transmigration of cancer cells in the microchannel array, and the amount of transmigrated cells drops significantly after the drug treatment. Statistics are done 24 hours after the start of the transmigration assay as plotted in Fig. 4.13. The application of 300 μM of MTA to the medium significantly reduces the transmigration of cells. The effect is more obvious in the narrow microchannels than that in the wide microchannels. The percentage of migrated cells dropped from 36 % to 30 % in 12 μm microchannel, 26 % to 14 % in 8 μm microchannel and from 11 % to 4 % in 4 μm microchannel.

As histone modifications can result in the changing of cellular transcription profiles and subsequently change the biological properties of the cancer cells, some histone-modifying reagents are now being tested in clinical trials as potential anti-cancer drugs [127]. The data from this chapter suggest that the anti-cancer properties of these drugs may be also related to additional effects on chromatin stability in resisting nuclear deformation. As histone modification and chromatin condensation facilitates nuclear reshaping and squeezing during the migration process, the ability of MTA to reduce condensation of chromatin fiber may impede the ability of the nucleus to undergo the structural changes required for the invasion of the metastatic cells through the small gaps in the extracellular matrix and epithelial syncytium.



(a) MDA-MB-231 cells not treated with MTA drug



(b) MDA-MB-231 cells treated with MTA drug

Figure 4.12: Fluorescent images of MDA-MB-231 cells (a) not treated with MTA drug (b) treated with MTA drug, migrating in the microfluidic chips. Nuclei of the cells are stained with fluorescent dye Hoechst 33423.

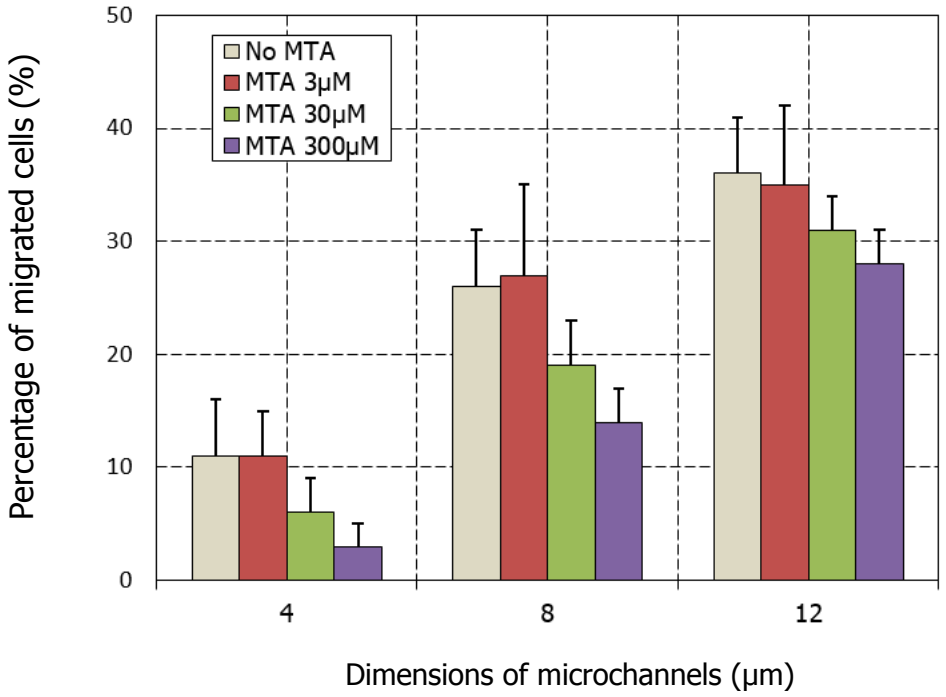


Figure 4.13: Statistical results of the percentage of MDA-MB-231 cells transmigrated through the microchannels after treatment with different concentration of MTA drugs.

In summary, the effect of histone-modulating drug MTA on the transmigration of cancer cells are studied. Experimental results show that MTA inhibits the transmigration of cancer cells in the microchannels in a concentration dependent manner. The findings confirm the importance of histone modification and chromatin condensation in cancer cell transmigration and will have implications in the design of new anti-cancer therapy.

4.6 Summary

In this chapter, the transmigrated cancer cells are analyzed by different biochemical methods to explore whether mechanical stress can change the chemical or biological properties of the transmigrated cancer cells.

In section § 4.1, the chromatins in cancer cell nucleus are stained with fluorescent dye DAPI, the results show that the transmigration of cancer cells induce chromatin condensation in their nucleus, which may be caused by the mechanical stresses imposed on the cells by the constricting geometries of the microchannels.

In section § 4.2, the histone proteins in cancer cell nucleus are immunostained with different fluorescent-labelled antibodies, results show that transmigration of cancer cells in the microchannels induce biochemical modifications on their histone proteins, more methylated histone and less acetylated histones in the nucleus of transmigrated cancer cells are detected.

In section § 4.3, the transmigrated cancer cells are isolated from the microfluidic chips and sub-cultured into other cell culture devices, the daughter cells harvested from propagation are analyzed, results show that chromatin condensation and histone modification induced by the transmigration of cancer cells can be inherited and kept in cells for certain period during the repeated cycles of cell division.

In section § 4.4, sub-cultured cancer cells are reloaded in to the microfluidic chips to test their transmigration capabilities. Results show that sub-cultured cells have higher transmigration capabilities than normal cancer cells, which may be related to the chromatin condensation and histone modification status in their nucleus.

In section § 4.5, pharmacological approaches to inhibit the transmigration of cancer cells in the microchannels are explored, results show that histone-modulating drug MTA inhibit the transmigration of cancer cells in the microchannels in a concentration-dependent manner, which may have implications in the design of new anti-cancer therapy.

In summary, it is proved through series of experiments that the physical deformations caused by mechanical stress from the confined geometries of the microchannels can change the chemical and biological properties of cancer cells. The combination of the microcluidic chips with biochemical analysis methods provides a good platform to study how the intracellular signaling pathways response to the stimuli from extracellular microenvironments.

CHAPTER 5

MICROFLUIDIC NEURAL CULTURE CHIP FOR AXON PATHFINDING

The extracellular signaling molecules locate near the tips of the growing axons are important for the development of individual neurons into functional neural networks *in vivo.* The signals can be sensed by neural growth cones and subsequently trigger cascade of intracellular molecular events which eventually affect the direction of the developing axons. To study the underlying mechanism of these important biological processes, neural culture devices which are able to present multiple molecular signals in a highly localized and systematic manner are required.

A neural culture chip with integrated microfluidic cell culture and protein patterning techniques is presented in this chapter. It is used to study the joint effects of both dissolved and substrate-bound molecular signals on the orientation of neural axonal growth. The design, fabrication and the experimental set-ups of the microfluidic chip will be illustrated, followed by the experimental results and discussions. The guidance effects of the immobilized protein patterns on the growth of neuron axons and the molecular mechanism behind the oriented axon growth will be studied.

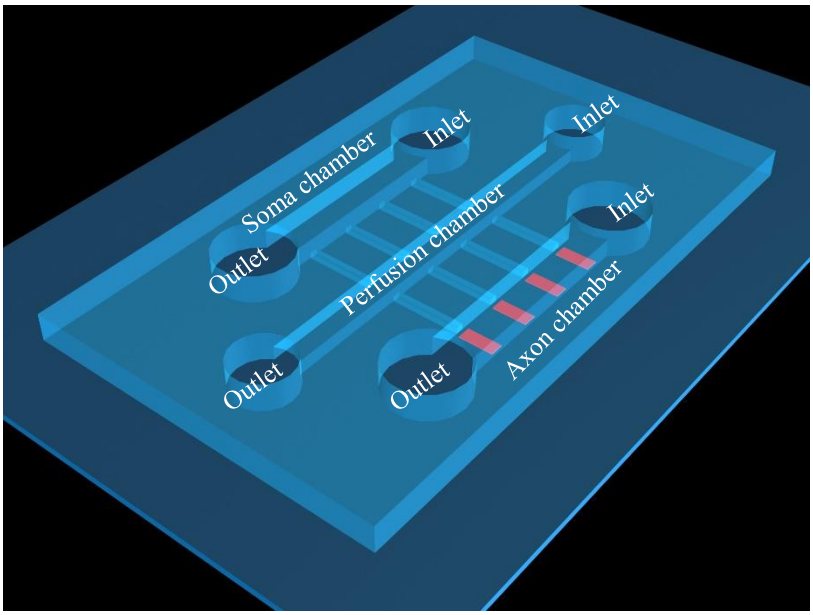
5.1 Design of microfluidic neural culture chip

Figure 5.1 shows a schematic illustration of the microfluidic neural culture chip. It consists of two major components, the first part is a cell culture chip with interior microstructures for compartmentalized neural culture and fluid isolation, the second part is a glass substrate patterned with signaling molecules for the orientation of neural axon growth.

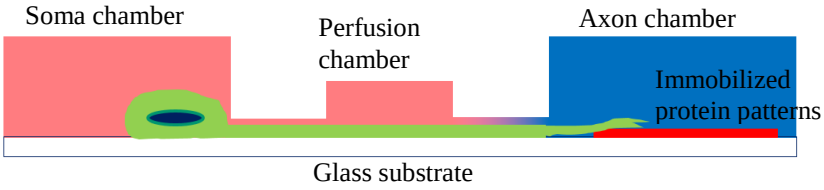
The two microchambers for neural culture are called as soma chamber and axon chamber, they are 10 mm long, 2500 μm wide, and 150 μm high. The central perfusion chamber has the same length and height as the two culture chamber but it is 200 μm in width. The central chamber is connected with two side chambers by series of perpendicular microchannels, each has a width of 10 μm, a length of 200 μm and a height of 5 μm, respectively.

When neurons are seeded in the soma microchamber, the axons are able to grow through the microchannel array toward the axon microchamber. The 5-μm height and 10-μm width are the limiting dimensions to avoid the somas of the neurons from entering the microchannel. In addition, the microchannels prevent liquids in neither chamber from flowing into adjacent chambers due to the high hydrostatic resistance in the channels. Thus, the major way for mass transportation between the chambers are though molecular diffusion in the liquid. To prevent the diffusion of dissolved chemicals from the one culture chamber to the other, perfusion can be set up in the central chamber with fresh cell culture medium. Thus, mass transportation between the two culture chambers can be limited to a minimum level and localized chemical treatment to either the soma or axon

side can be achieved.



(a)



(b)

Figure 5.1: Schematic illustration of the neuron culture chip. (a) overview of the chip (b) working principle. Neurons are seeded in the soma chamber, the axons can grow through the microchannel array toward the axon chamber. The perfusions in the central chamber limits the diffusion between the soma and axon chamber. Proteins patterns (red color) are immobilized on the substrate on the glass bottom of the axon chamber for the guidance of axon growth.

The other major component of the chip is the patterned glass substrate for the guidance of neural axon growth. Specific biomolecules are immobilized on the glass into defined patterns using microcontact printing method. When the axons of cultured neural cells extend from soma chamber to the axon chamber, they can respond to the immobilized biomolecules and adjust their growth routes according to the geometry information provided by the specific patterns. The growth profile of the neural axons can be further modulated by the application of chemical reagents in the axon or soma chamber.

5.2 Fabrications and experimental procedures

5.2.1 Fabrication processes

The fabrication process for the microfluidic neural culture chip is shown in Fig. 5.2. The soft lithography for the fabrication of PDMS microfluidic chip and the microcontact printing for protein patterning on the glass coverslip are done separately and the individual products are assembled to form the integrated neural culture chip.

Microfluidic chip

The microfluidic chip is fabricated by using standard soft-lithography techniques. The master is fabricated on the silicon wafer using a two-step photolithography method. Firstly, a 5-µm thin layer of SU-8 photoresist (SU-8 10, MicroChem) is spin-coated on a silicon wafer (CEE 200, Brewer Science). After soft baking, it is exposed with the first chrome mask, which patterns the microchannels, and followed by the post exposure bake. After development, a second 100-µm thick layer of SU-8 (SU-8 100, MicroChem) photoresist is spin-coated on top of the first layer and exposed with a second mask to pattern the neural culture and perfusion chambers. PDMS (Sylgard 184, Dow Corning) prepolymer and curing agent mixture (10:1, v/v) are poured over the silicon master, degassed and baked for 2 hours at 75 °C and then peeled off. The inlet and outlet holes are punched manually using a Harris uni-core sampling puncher.

Substrate preparation and protein patterning

Treatment of glass with strong acidic solutions can render the surface clean and highly hydrophilic, which is important for the attachment of the biomolecules. Before patterning proteins, glass coverslips (25 mm, Fisher Scientific) are cleaned in Piranha solution (H_2SO_4 mixed 3:1 with 30% hydrogen peroxide (Sigma)) at room temperature for 30 minutes, rinsed extensively with deionized water, and dried with nitrogen gas blow.

Proteins are patterned at specific location of each coverslip through microcontact printing. The PDMS stamps are fabricated using same soft lithography method as for the fabrication of the microfluidic cell culture chip. The stamps containing designed geometry features are soaked in 100 μg/ml laminin or 100 μg/ml type IV collagen (GE Healthcare) mixed with 0.005% FITC-BSA (Invitrogen) in PBS for one hour at 37 °C. The stamps are washed with deionized water and dried by nitrogen gas blow and placed in conformal contact with piranha-treated glass coverslip for 10 seconds. The stamps are then removed and the glass coverslip is rinsed extensively with deionized water, and dried with nitrogen gas blow.

Assembly

Both of the PDMS microfluidic chip and patterned coverslip are further plasma treated for 15 seconds, a piece of plain PDMS is put on top of the stamped region on the glass coverslip during the treatment to protect the attached proteins. The treated coverslips and PDMS chip are assembled under microscope to ensure that the exits of the microchannels are close to the protein patterns.

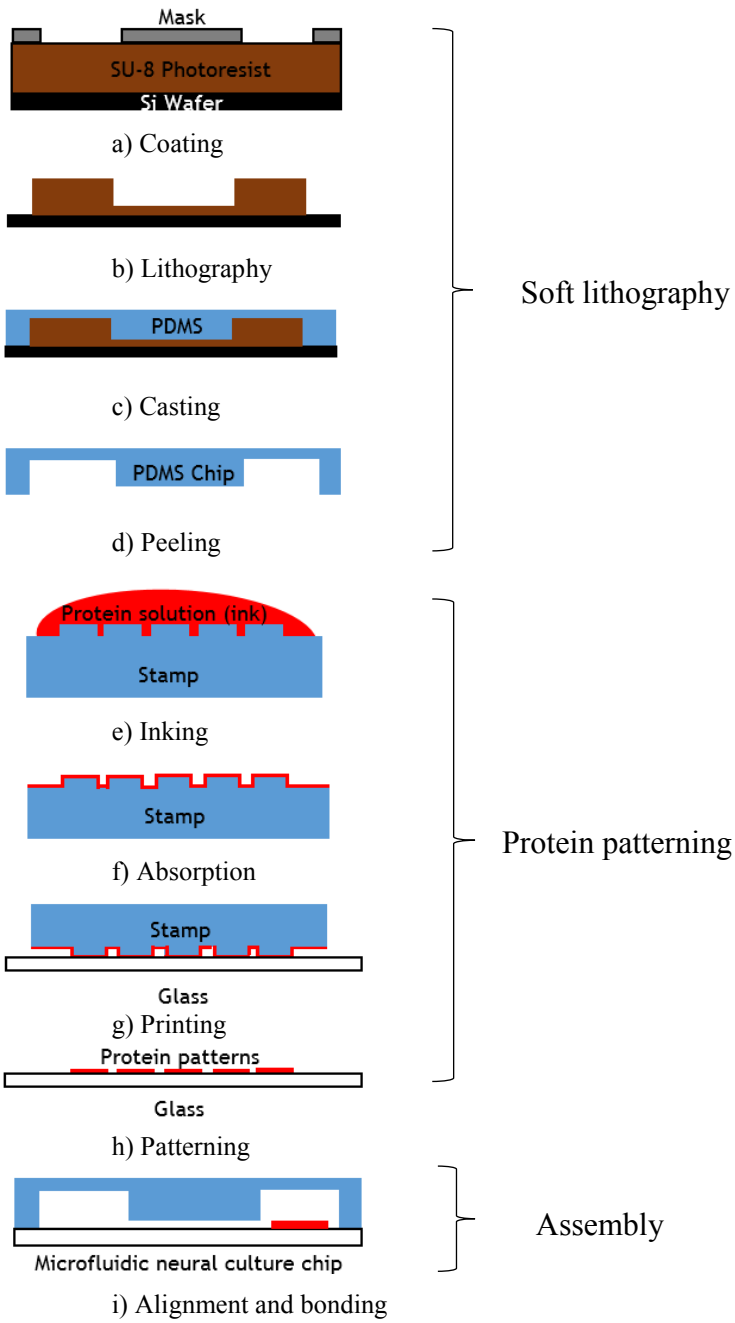


Figure 5.2: Fabrication process of the microfluidic neural culture chip

5.2.2 Neuron culture

Primary cultures of E18 rat cortical neurons are prepared using standard protocols. Briefly, cortexes are dissected from E18 rat embryos in calcium and magnesium free Hanks' balanced salt solution (HBSS). Then the cortical neurons are dissociated from the cortex tissue by using a papain dissociation kit (Worthington Inc.) based on the manufacturer's protocols. The dissociated cells are resuspended in a chemically defined medium of B27, glutamate, and glycine in neurobasal medium (Invitrogen) at a concentration of 1×10^7 cell/ml and injected into the microfluidic chip. Neurons are fed twice a week by refreshing half of the medium.

5.2.3 Immunofluorescence staining and data analysis

PDMS microfluidic chip is removed from the glass coverslip and the cells on the glass are gently rinsed with PBS (pH 7.4) and fixed with 4% paraformaldehyde. After rinsing, cells are incubated with blocking solution (0.1% Triton X-100 and 10% bovine serum albumin in PBS) for one hour at room temperature. Cells are then incubated for 1 hour at 37 °C with polyclonal anti β-Tubulin (type-III) antibody (Abcam) diluted 1 : 500 in blocking solution, after rinsing in PBS, cells are incubated with goat anti-rabbit Alexa 488-conjugated IgG (1 : 1000; Invitrogen) for 1 hour at 37 °C.

The neurons are imaged using Nikon Ti-E microscope. Objective lenses used for imaging, including Nikon Plan Fluor 10× and 20× dry lenses and a 40× oil lens, with numerical apertures of 0.3, 0.75 and 1.3, respectively. The captured images are analyzed by NIS-AR Elements software.

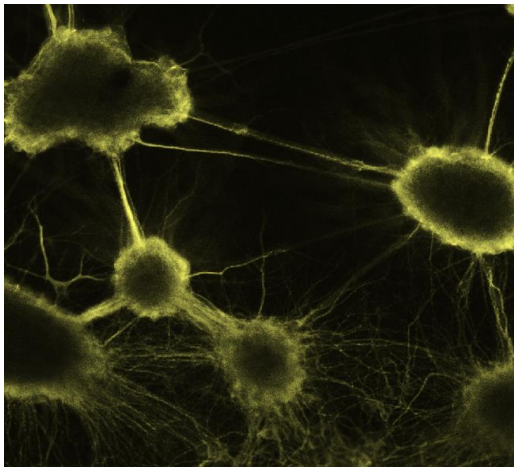
5.3 Experimental results and discussions

5.3.1 Optimization of neuron culture in microfluidic chips

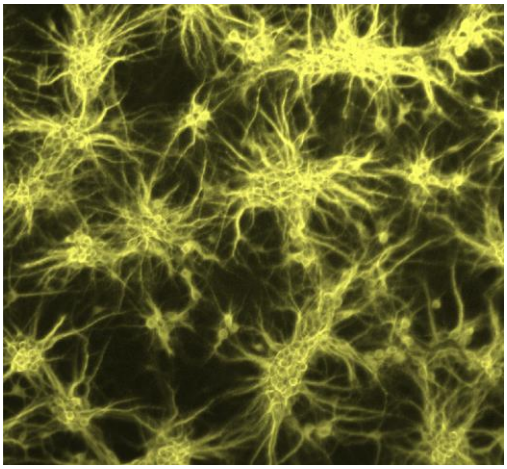
Neuron culture *in vitro* is challenging since the primary cells isolated from mouse embryo have more stringent requirements on environmental conditions than other commonly used cell lines. To ensure healthy neuron cultures, series of optimization works are carried out to improve the conditions in the microfluidic chips.

Coating of glass substrate

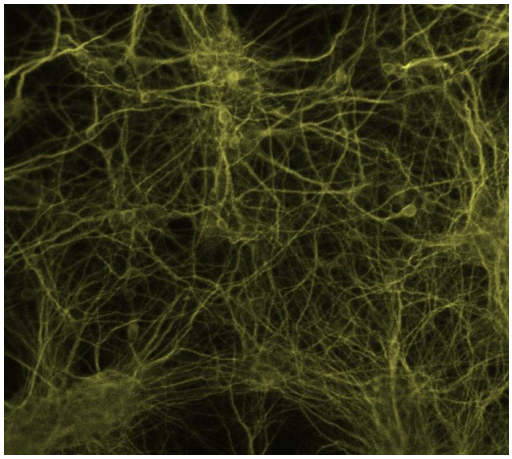
Anchorage of neurons to the glass surface in the microfluidic chip is important for their survival and growth. It can be enhanced through surface coating. The most commonly used coating reagents are positively charged polymers such as poly-L-lysine (PLL) or biologically purified adhesive molecules from extracellular matrix (ECM) such as collagens and fibronectins. To find out the best coating protocol for the culture of cortical neurons in the microfluidic chip, different coating solution is filled into the microchambers after assembly. After incubation at 37°C in a humid environment for 12 hour, the coating solution is washed off with deionized water. Cortical neurons are then loaded into the chip.



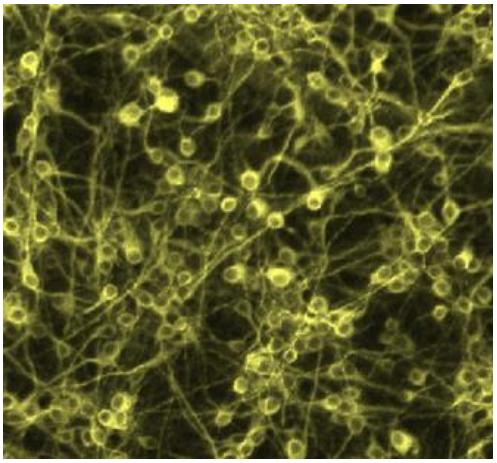
(a) Non-coated



(b) PLL coated



(c) Fibronectin coated



(d) PLL+Fibronectin coated

Figure 5.3: Fluorescent images of mouse cortical neurons growing on glass surfaces coated with different adhesive molecules after 7 days culture *in vitro*. Cells are stained with anti β-Tubulin (type-III) antibodies. (a) Neurons grow on non-coated glass surface. (b) Neurons grow on PLL coated glass surface (c) Neurons grow on fibronectin coated glass surface (d) Neurons grow on PLL plus fibronectin coated

Growth patterns of mouse cortical neurons on glass surfaces coated with different materials are shown in Fig. 5.3. Neurons growing on the non-coated glass can hardly attach to the surface and tend to clump together to form large neuro-spheres. Most of the neuron axons are bundled together and form cable-like connections between different neuro-spheres. Results show that single coating of glass surface with either 1mg/ml PLL or 50 μg/ml fibronectins can significantly improve the neuron attachment. The cells attached to the coated surface can form smaller clusters and more connections between individual neurons are observed. Most healthy neuron culture is achieved on the surface coated with combined formula of 0.5mg/ml PLL plus 10 μg/ml fibronectins, on which most of the neurons are separated from each other and attach firmly onto the glass substrate. Extensive neural networks are formed after 7 days of culture *in vitro*.

PDMS pretreatment

It has been reported that 5% (w/w) of bulk PDMS is un-crosslinked oligomers, which can leak from the materials into cell culture medium and impede the growth of primary neurons [128]. Previous studies showed that these oligomers can be removed by extraction through soaking of PDMS in a series of organic solvents, and the treatment procedures have shown to influence the attachment and proliferation of human endothelial cells [129]. It is hypothesized that extracting PDMS chip with organic solvents prior to assembly will also improve the survival of primary cortical neurons in the microfluidic chip.

To test this hypothesis, PDMS chip is treated sequentially in following organic

solvents with continuous stirring: pentane for 7 hours, xylene isomers plus 98.5% ethylbenzene for 2 hours, xylenes for 16 hours, ethanol for 24 hours. After extraction with the organic solvents, the chips are washed in sterile deionized water overnight. The extracted chips are then dried at 70 °C overnight before assembly with the glass substrate. The viabilities of neurons inside the extracted and non-extracted PDMS chips after several days of culture are assessed using calcein AM and ethidium homodimer live/dead stain (Molecular Probes). As shown in Fig. 5.4, the survival rate of neurons in the extracted chips are significantly higher than in the non-extracted chips, especially after long period of culture, the majority of the neurons in the non-treated chip cannot survive for longer than 14 days, whereas in the treated microfluidic chip most of the cells can remain healthy up to 21 days.

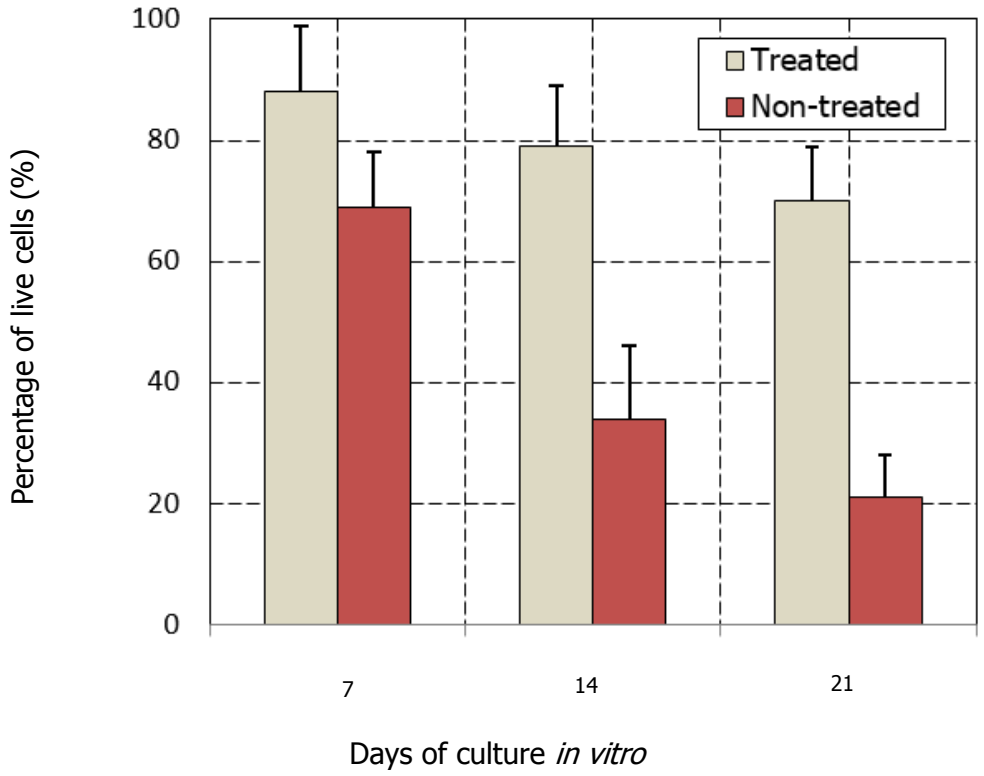


Figure 5.4: Growth profile of neurons in extracted and non-extracted PDMS chips

5.3.2 Compartmentalized neuron culture

Figure 5.5 shows the culture of rat cortical neurons in the microfluidic chip for 14 days under optimized conditions, the PDMS cell culture chip has been pretreated by organic solvent extraction and the glass surface in the soma chambers are coated with PLL plus fibronectins. The cells are immunostained with anti β-Tubulin (type-III) antibody. It can be seen that due to the size restriction of the microchannels, the cell bodies (somas) of the neurons remain exclusively in left (soma) microchamber. After the cells attach to the coated glass surface, they are able to extend their neurites through the first microchannel array. Pioneer neurites are observed in the central perfusion chamber 2 days after the start of culture, they can be either dendrites or axons. Without the guidance of the microchannels, the neurites extend themselves in a random manner in the perfusion chamber, when they reach the entrance of second microchannel array, some of them are able to extend through and finally reach the right (axon) chamber. Since the maximum length of the cortical dendrites are less than 400 μm, the neurites extended to the right chambers are purely axons. Abundant axons can be seen in the axon chambers after 14 days of culture as shown in Fig. 5.5.

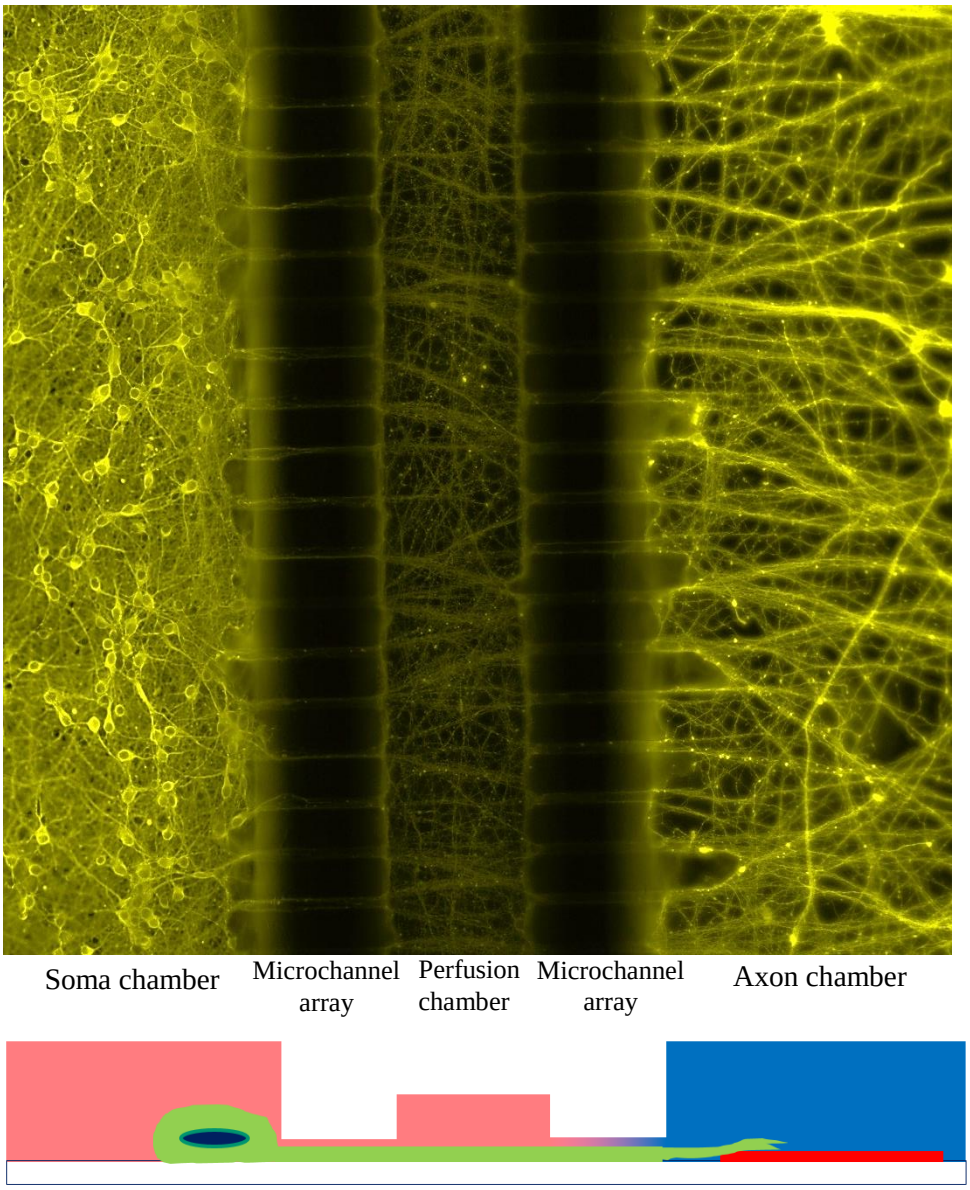
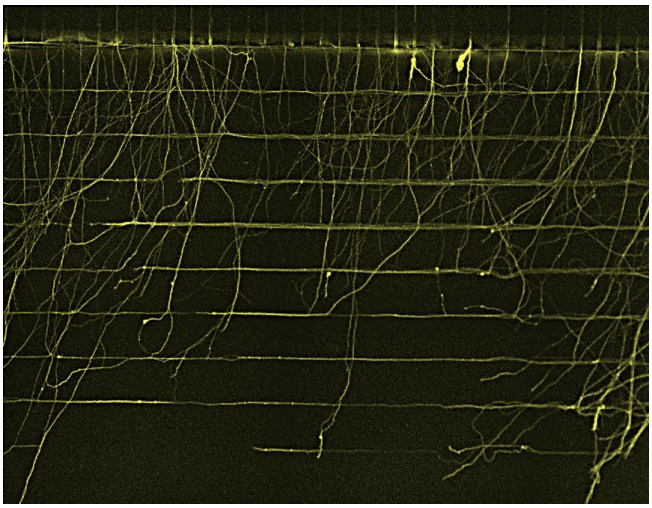


Figure 5.5: Fluorescent images of rat cortical neurons cultured in the microfluidic chip. Neurons are stained with anti β-Tubulin (type-III) antibody.

5.3.3 Orientation of neuron axon growth

The immobilization of biochemicals on glass substrate through microcontact printing can be used to guide the growth of neuron axons. Either laminin or type IV collagen are printed in line shapes onto the glass coverslip pre-coated with PLL. When the axons of the cultured neurons extend into the axon chamber from the exit of the microchannels, they first grow randomly on the PLL coated glass surface without patterned proteins, when the tips of growing axons encounter the printed protein lines, the receptors on the growth cones can sense the immobilized proteins and trigger intracellular signaling events. The axons then make decisions whether to follow or ignore the directions of the protein lines.

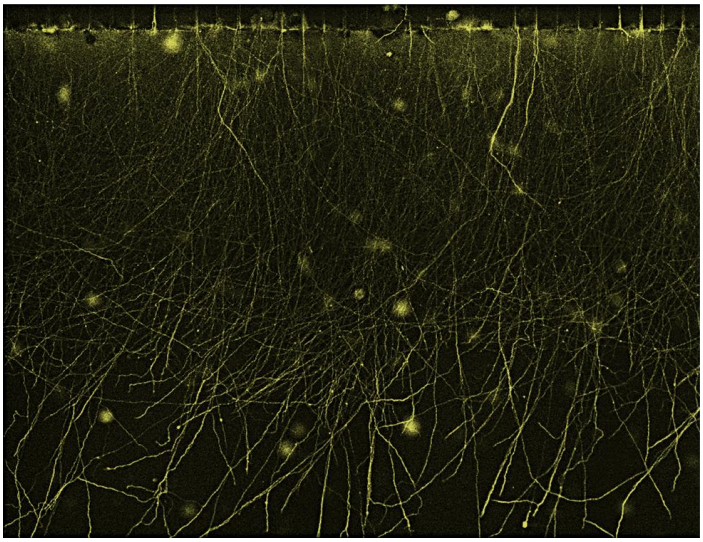
As shown in Fig. 5.6, axons encountering lines of laminins redirect and follow these lines in contrast to the intervening regions of PLL. In contrast, axons encountering lines of type IV collagen are not similarly guided, the majority of the axons tend to ignore the patterned lines and the directions of axon extension are not affected as shown in Fig. 5.7.



(a) Neural axons grow on microcontact-printed laminin lines.

(b) Fluorescent images of microcontact-printed laminin lines.

Figure 5.6: Growth pattern of neuron axons on glass surface with microcontact-printed laminin lines. Neurons are stained with anti β-Tubulin (type-III) antibody. (a) Fluorescent image of neural axons grow on microcontact-printed laminin lines. (b) Fluorescent image of microcontact-printed laminin lines.



(a) Neural axons grow on microcontact-printed type IV collagen lines.

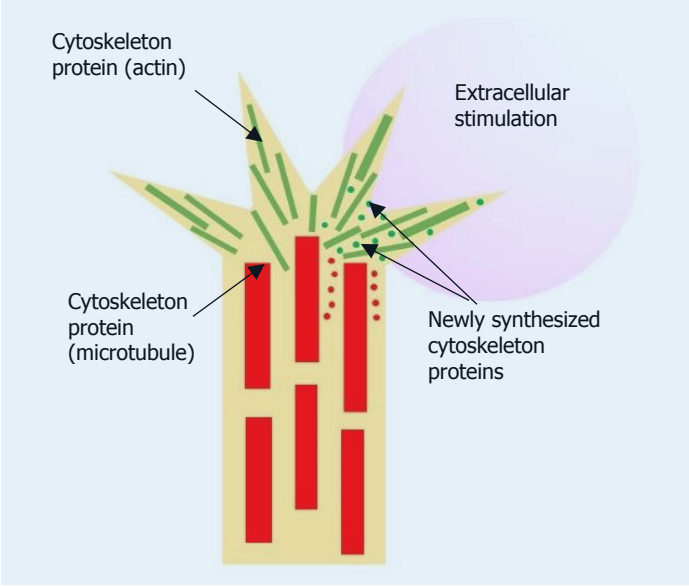
(b) Fluorescent images of microcontact-printed type IV collagen lines.

Figure 5.7: Growth pattern of neuron axons on glass surfaces with microcontact-printed type IV collagen line. Neurons are stained with anti β-Tubulin (type-III) antibody. (a) Fluorescent image of neural axons grow on microcontact-printed type IV collagen lines. (b) Fluorescent image of microcontact-printed type IV collagen lines.

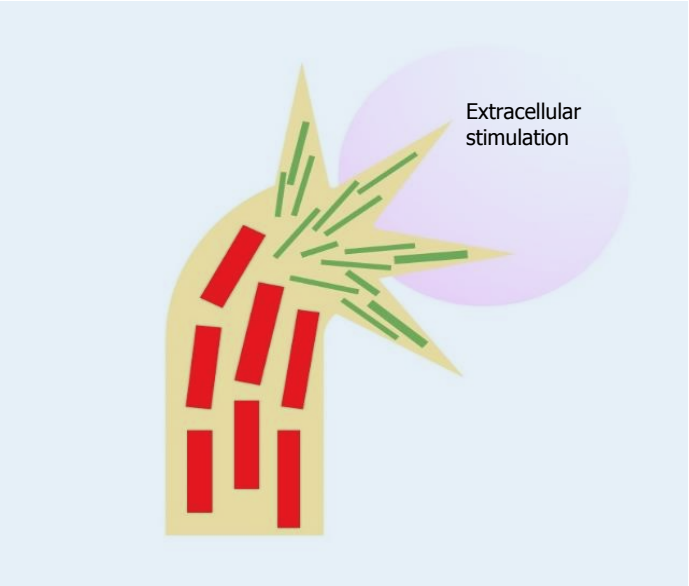
5.3.4 Modulation of axon guidance by localized drug treatment

Nascent proteins required for neuron growth can be produced in both cell body and axons, although proteins produced in axons only count for a small portion, growing evidences have shown that they may play important roles in the response of neural axons to extracellular guidance cues. It has been reported that axonal protein synthesis may supply cytoskeletal proteins needed for growth cone motility [130, 131]. As shown in Fig. 5.8, the newly synthesized cytoskeleton proteins are assembled onto the existing backbones facing the source of extracellular stimulation, and the axons can subsequently extend toward the directions of the stimulation source.

In this section, the role of local protein synthesis in the growth of neural axons are studied using the microfluidic neural culture chip. The local protein synthesis and cytoskeleton remodeling of the neurons cultured in the microfluidic chips are interfered through localized drug treatment and the growth profile of neural axons on the microcontact-printed protein laminin lines are studied.



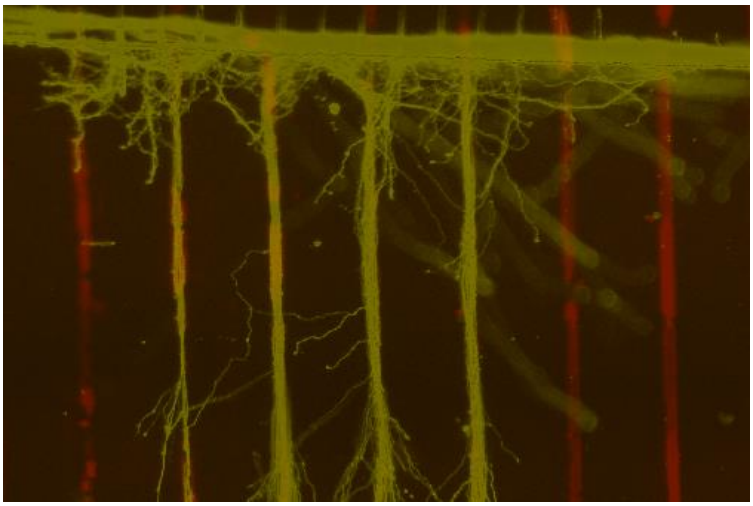
(a) Stimulation and local protein synthesis



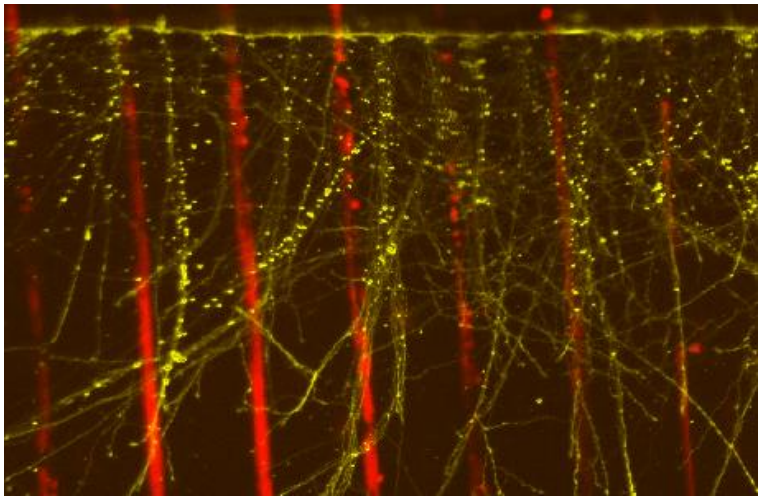
(b) Axon turning

Figure 5.8: Illustration of the proposed molecular mechanism for axonal pathfindings in neuron. (a) Extracellular stimulations can trigger the local synthesis of cytoskeleton proteins. (b) The newly synthesized proteins are assembled and eventually affect the direction of neural axon growth.

After 5 days of neuron culture, when neuron axons start to extend into axonal chamber, protein synthesis inhibitor cycloheximide [132] or cytoskeleton destabilizer nocodazole [133] (sigma) are added into either axon chamber or soma chamber. Meanwhile, perfusion is set up in the middle perfusion chamber to prevent the drugs from diffusing into opposite culture chamber. After further culture for 48 hours, the cells are immunostained with fluorescent antibodies and their growth patterns on the glass substrate are analyzed.



(a)



(b)

Figure 5.9: Modulation of axon growth on microcontact-printed laminin proteins by localized drug treatment. (a) The axons are restricted to the printed laminin lines when no drug are added. (b) The orientations are disrupted when protein synthesis inhibitors are added to the axonal chamber.

Figure. 5.9 shows the modulation effect of localized drug treatment on neural axon growth. When 500 ng/ml of protein synthesis inhibitor cycloheximide is applied to the soma chamber, majority of the cells detach from the glass coverslip and the cells are no longer alive after 48 hours. On the contrary, neurons are able to remain healthy after cycloheximide is applied to the axon chamber, the distance that the axons have extended over 48 hours after adding the drugs are close to the cells without drug treatment. It is observed that applying of cycloheximide to the axonal chambers significantly changed the pattern of axonal growth on microcontact-printed laminin lines. To quantify the response of axons to patterned protein lines under different experimental conditions, a guidance index is used to calculate the distribution of neural axons on the patterned protein substrate. The guidance index can be expressed as:

$$\text{Guidance index} = \frac{A}{\quad} \tag{5.1}$$

where A is the yellow fluorescent intensity of neuron axons growing on a printed laminin strip and B is the yellow fluorescent intensity of neuron axons growing on an adjacent background stripe of same area without printed laminin proteins. When no drugs are added to the axonal chamber, the guidance index is close to 1, indicating a strong preference of axonal growth on the laminin strip. In contrast, in the chambers filled with cycloheximide, axon growth shows more scattered patterns. They are no longer restricted onto the laminin strips and the guidance index dropped significantly to 0.73 as shown in Fig. 5.10.

Cytoskeleton destabilizer nocodazole can block the assemblies of newly

synthesized cytoskeleton proteins which are produced either locally in the axon chamber or distantly in the soma chamber onto the backbones of the existing axons. When 200 ng/ml of nocodazole are added to axon chambers, the axons extend at a much slower rate than those cells without drug treatment, and the growth of axons shows a more spread pattern than cycloheximide treated cells with a lower guidance index of 0.54. On the contrary, when the same amount of the drug are added directly to soma chamber, the cells are not able to survive for longer than 48 hours.

The experimental results suggest that the majority of the proteins which are required for axon extension come from neural soma, proteins synthesized locally in axon may not be important for axon extension but important for the directions of their growth.

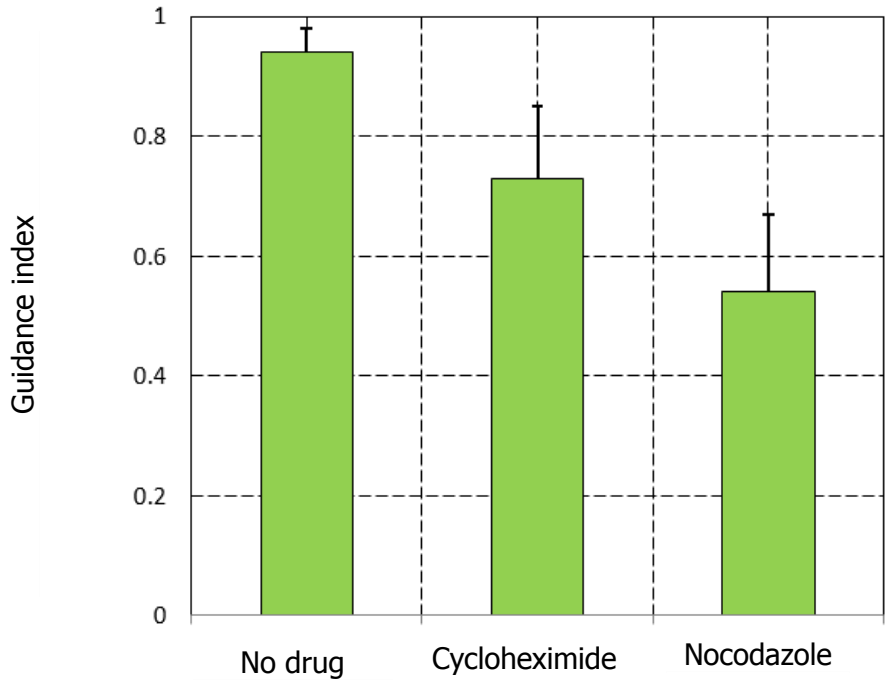


Figure 5.10: Effect of localized drug treatment on the guidance effect of laminin to axonal growth.

5.4 Summary

In this chapter, a microfluidic neural culture chip for the study of axon pathfinding is presented. The design of the microfluidic chip is demonstrated in section §5.1, it consists of cell culture chip with interior microstructures for compartmentalized neural culture and glass substrate patterned with signaling molecules for the orientation of neural axon growth. The fabrication of microfluidic chip and the experimental set-up is shown in section §5.2. The experimental results are shown in section §5.3, the somas and axons of neurons cultured in the microfluidic chip can be polarized into different fluidically isolated environments for long period, meanwhile the extensions of the axons can be guided by proteins immobilized on the glass substrate into specific patterns. The orientated axon growth can be further modulated by localized drug treatment. Studies on the underlying molecular mechanism shows that these processes are closely associated with the proteins synthesized locally in the tips of growing axons.

In summary, the microfluidic neural cell culture chip enables the study of intracellular signaling on a localized basis, it will be of useful in many aspects of cell biology research, especially for those focusing on cells with extended morphology such as neural cells and some epithelial or endothelial cells.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Understanding the functional consequences and the molecular mechanisms of the micro-environmental extracellular stimuli on the growth and development of different cells is a great challenge in current biology research. In this PhD project, two *in vitro* cell culture devices were developed via microfbrication technologies, which provides entirely new levels of controls over the cell culture microenvironment. The applications of the developed devices in cancer and neurobiology researches were demonstrated, specifically for the study of cancer metastasis and neural axonal pathfinding.

The two new cell culture chips utilized different features of the microfabricated devices for biology researches. For the microfluidic transmigration chip demonstrated in chapter 3, it takes advantage of the microstructure dimensions, which are comparable to the sizes of tissue capillaries and single cancer cells. Therefore it is suitable for the study of cell confinement and deformation. The transparent optical qualities of the device also allows live imaging of cancer cells during their transmigration process in the artificial capillaries. Studies on the cellular dynamics showed that deformation of the stiff cell nucleus were the most time-consuming steps during the transmigration process. The physical restrictions not only changed the morphology of the cells, but also significantly affect their migration velocity. It cannot be compensated by the increase of

chemoattractant which provide directional cues and driven power for migration, implicating that the mechanical stress might affect the cellular motors responsible for cell migration.

The microfluidic transmigration chip is also compatible with other molecular biology assays like immunofluorescent staining and western blot analysis, thus the intracellular molecular signaling events during the transmigration process can be studied. In chapter 4, further studies on the molecular contents and biological properties of the transmigrated cells showed that the mechanical stress imposed on the cancer cells by the confined geometries could induce biochemical modifications on their nuclear histone proteins and the elevation of their transmigration capabilities. Blocking the histone modification by specific drug can inhibit the transmigration of cancer cells in the microchannel, which might have implications on cancer prevention and treatment. The chip can also be used to access cell deformability, which is a potential prognostic marker for cancer diagnosis. Results suggested that the microfluidic cancer cell culture chip is not only useful for the fundamental research in cancer science, but also for cancer diagnosis and development of new anti-cancer therapy.

The neural culture chip presented in chapter 5 mainly utilizes the capabilities of the microstructures in fluid isolation for compartmentalized neural culture and capabilities of patterned substrates in the guidance of cell growth. To explore the molecular mechanisms and signaling pathways activated locally by multiple guidance cues during axon growth, microfluidic cell culture and protein patterning techniques are integrated to mimic the complexity in the *in vivo* cellular microenvironments. The device

combines the functions of traditional stripe assay and Campenot chambers, meanwhile proved much more precise manipulation of the environmental conditions. Well isolated fluidic environments in both axon and soma compartments enables the applying of pharmacological reagents to different sub-domains of a neuron, which will be useful in the investigation of many local events in axon, such as electric signal transmission, axonal transport, and biomolecule synthesis. Using the integrated neural culture chip, the molecular mechanisms responsible for the oriented growth of neuron axons on the immobilized protein patterns were studied, results showed that it was closely associated with the proteins synthesized locally in the tips of growing axons.

In conclusion, the capabilities of micro-fabricated cell culture devices in mimicking complicated *in vivo* cellular microenvironments were demonstrated through two novel microfluidic chips for cancer and neuroscience researches. With the new techniques and research methodologies available, more fundamental questions regarding the structures and functions of individual cells can be addressed and it will lead to a deeper understanding of many basic biological processes and better medical treatments of related diseases.

6.2Recommendations

While some promising results have been obtained, additional developments can be made on the devices to improve the variety, accuracy and efficiency of the experiments. Meanwhile researches on cancer metastasis and neural axon pathfindings can be pursued further for deeper insights of these important biological processes. The recommendations for future works are summarized as follows.

a) Current microfluidic transmigration assay mainly focus on the effects of the geometry constriction, which is only one of many important features of the extracellular environment cancer cells encountered during their metastasis *in vivo*. For better mimicking of the microenvironment, hydrogels dissolved with macromolecules from the extracellular matrix such as proteins, lipids and proteoglycans can be used to fill the microchannels in the transmigration chip, and some important cellular mechanisms such as proteolysis can be studied.

b) Current designs of the transmigration chip only have straight microchannels with universal diameter through the whole length. Since the actual structures of the tissue capillaries cancer cells facing *in vivo* are much more complicated, it will be good to incorporate different geometries such as angles and curves into the designs and study the migration profile of cancer cells.

c) Results showed that physical deformations caused by the mechanical stress can induce biochemical modifications on cancer cells' nuclear histone proteins. Since histone modifications are closely associated with diverse biological processes such as gene regulation, DNA repair and cell dividing. Further studies at cellular and molecular

biology level may lead to the discovery of other important consequences. This will require the extraction of DNA, RNA and protein contents from the migrating cells on the microdevices, gene transcription profiles can be analyzed by relevant molecular biology assay like RT-PCR and proteomic analysis.

d) Current studies on the integrated neural culture chip mainly focus on the effects of chemical cues on the guidance of axon growth. Besides chemical cues, neural axons also sense and respond to other types of stimuli from their microenvironments such as mechanical stress and topological information on the substrate. It will be good to improve the design of current device so that those different types of environmental cues can be presented to neuron growth cones. One easy method to apply mechanical stress is through the shear forces induced by the perfusion of medium in the microfluidic device. Topology features can also be created on the substrate using fabrication techniques such as glass etching.

e) Since the microfluidic neural culture chip is able to separate cells into isolated environments, co-culture of different groups of neurons can be set up. With the established axon guidance techniques, the connections (synapses) can be established at designated location on the device. Through localized stimulation on individual groups of the neurons, systematic study of the signal transduction with the neural networks can be performed.

Résumé long

Dans ce projet de thèse, deux dispositifs de culture cellulaire *in vitro* sont développées via des technologies de microfabrication, lesquelles fournissent un niveau de contrôle complètement nouveau par rapport au micro-environnement *in vitro* de culture cellulaire. Les applications des dispositifs développés sont démontrées dans la recherche sur le cancer et en neurobiologie, en particulier pour l'étude fondamentale de métastases du cancer et de l'orientation de la croissance axonale de neurones.

La thèse est organisée en six chapitres.

Le chapitre 1 est la section d'introduction, il couvre la motivation, l'objectif, et contributions majeures de cette thèse. Nous commençons par expliquer les raisons qui nous ont conduits à développer la recherche effectuée. Nous y présentons les objectifs poursuivis, puis les principales innovations ainsi que résultats les plus saillants. Dans le chapitre 2, une revue de la littérature sur les connaissances de base liées à cette thèse est présentée. Elle couvre l'introduction aux dispositifs de culture cellulaire micro-fabriqués, un résumé de leurs principales caractéristiques et les techniques de fabrication les plus couramment utilisées. Nous fournissons également des informations de base sur les pathologies du cancer ainsi que sur le développement de cellules nerveuses, suivi d'un aperçu des méthodologies actuelles utilisées dans ce type de recherches. Nous entamons ensuite une discussion sur les problèmes répertoriés ainsi que les solutions possibles qui pourraient être apportées par la mise en œuvre de nouveaux outils expérimentaux façonnés par des techniques de microfabrication.

Dans le chapitre 3, une puce microfluidique pour l'étude de la transmigration des cellules cancéreuses est présentée. Tout d'abord, nous introduisons la conception et les

principes de fonctionnement de la puce microfluidique. Nous présentons également le procédé de fabrication du microsystème puis nous développons les protocoles mis en œuvre pour réaliser les expériences de transmigration sur cette puce, et présentons ensuite les premiers résultats expérimentaux. Différents types de cellules cancéreuses sont testées au moyen de la puce développée. Les profils de transmigration des cellules cancéreuses sont examinés en fonction de différents paramètres : géométrie de la conception et conditions expérimentales.

Dans le chapitre 4, des analyses biochimiques complémentaires et plus détaillées sont effectuées sur les cellules cancéreuses transmigrées. L'accent est mis sur les composants du noyau de la cellule, y compris les chromatines et des protéines histones. La condensation de la chromatine et l'état de la modification des protéines histones sont analysés à travers différents dosages biochimiques par techniques d'immunofluorescence et *western blot*. Les cellules cancéreuses transmigrées sont isolées des micro-puces et propagées dans un dispositif standard de culture cellulaire. Les cellules filles issues de cette propagation sont également analysées afin de vérifier si elles peuvent hériter des propriétés chimiques et biologiques des cellules mères. La possibilité d'utiliser des médicaments capables de moduler la chromatine est ensuite explorée en vue de prévenir les métastases du cancer. Certains médicaments ayant des capacités de blocage de changements de structure de la chromatine sont appliqués sur les cellules cancéreuses et leurs profils de transmigration sont étudiés.

Dans le chapitre 5, nous présentons une seconde puce microfluidique de culture, adaptée cette fois-ci aux cellules neuronales ; cette puce intègre à la fois la capacité de culture cellulaire microfluidique mais aussi une technique d'impression de motifs

géométriques de protéines. Tout d'abord, la conception et les principes de fonctionnement de la puce sont illustrés. Les méthodes de fabrication du microsystème et les protocoles expérimentaux sont ensuite détaillés, suivis par la présentation des résultats expérimentaux. La culture cloisonnée de neurones corticaux embryonnaires de souris est démontrée. L'effet de l'orientation des motifs de protéines immobilisées sur la croissance des axones des neurones est étudié. Le mécanisme moléculaire sous-jacent à la croissance orientée de l'axone est également exploré à travers un traitement médicamenteux localisé.

Le chapitre 6 conclut et résume les principales contributions de cette thèse suivie par les recommandations pour les travaux futurs.

Chapitre 1 Introduction

Les signaux de l'environnement extracellulaire hétérogène couvrent de grandes échelles spatiales et temporelles. En raison de limitations techniques, la plupart des plateformes existantes pour la culture cellulaire *in vitro* ne sont capables de présenter que des indices environnementaux simples à des grands groupes de cellules. Bien que de nombreuses recherches aient déjà été effectuées, les informations sont limitées quant aux réponses cellulaires à des signaux extracellulaires multiples exercés sur une cellule unique ou au niveau sub-cellulaire.

Les principaux objectifs de cette thèse consistent à développer et à expérimenter de nouveaux dispositifs de culture *in vitro* de cellules cancéreuses et de neurones en mettant en œuvre des techniques de microfabrication. Ces nouveaux dispositifs seront capables de mimer le micro-environnement rencontré par les cellules cancéreuses individuelles au

cours de leur métastase ou par des cellules neuronales au cours de leur croissance. Les effets fonctionnels des facteurs micro-environnementaux sur la croissance et le développement de cellules cancéreuses et de neurones seront systématiquement étudiés.

Les principales contributions de cette thèse sont résumées comme suit: a) une puce microfluidique de transmigration à base de polydimethylsiloxane (PDMS) a été développée. Il incorpore des micro-canaux structurés pour imiter les capillaires tissulaires le long du trajet de la métastase des cellules cancéreuses. L'effet d'un confinement géométrique sur le profil de migration de différentes cellules cancéreuses a été étudié. b) A travers une série d'analyses biochimiques, il a été prouvé que la contrainte mécanique imposée aux cellules cancéreuses pouvait induire une modification biochimique de leurs composants nucléaires. La cellule cancéreuse modifiée développe alors des capacités métastatiques plus élevés que les cellules non stressées. Le blocage de cette modification par des approches biochimiques pharmaceutiques pourrait alors inhiber la métastase des cellules cancéreuses. c) Une puce microfluidique de culture de neurones a été développé, les somas et les axones des neurones en culture dans le microsystème peuvent être polarisés dans différents environnements fluidiques isolés sur une longue période, et l'extension des axones des neurones ont été guidés par des protéines immobilisées sur le substrat de verre selon des motifs spécifiques. La croissance axonale orientée peut en outre être modulée par un traitement médicamenteux localisé. Les études sur le mécanisme moléculaire sous-jacent ont révélé que ces processus ont été étroitement associés à des protéines synthétisées localement dans les extrémités d'axones en croissance.

Chapter 2 Etat de l'art

Dans ce chapitre, des informations de base relatives au sujet de thèse sont passées en revue et discutés lorsque nécessaire. Le chapitre se compose de trois parties, la section § 2.1 porte sur les technologies de microfabrication et leurs applications dans la recherche en biologie; les techniques de microfabrication ont été initialement développées pour la fabrication de dispositifs micro-électroniques à base de silicium. Lorsqu'elles sont utilisées dans le développement de micro-dispositifs basés sur des cellules, elles sont capables de créer des microstructures à deux dimensions (2D) ou à trois dimensions (3D) avec une précision à échelle micrométrique, et incorporer des matériaux différents avec des propriétés chimiques ou physiques distinctes. Les facteurs micro-environnementaux au sein des structures, telles que les conditions fluidiques, les transports de masse et les conditions de surface, peuvent également être contrôlés avec précision. Les méthodes de fabrication les plus communément utilisées incluent la lithographie douce, la fonctionnalisation de surfaces et la micro-impression par contact.

Les deux sections suivantes de ce chapitre introduisent deux champs de recherche en biologie cellulaire dans lesquels les techniques de microfabrication sont et continueront vraisemblablement à être utilisées pour résoudre des problèmes de recherche scientifique. La section § 2.2 porte sur la biologie du cancer, y compris l'introduction de connaissances de base relatives aux maladies du cancer, des cellules tumorales et de leurs métastases. La transmigration de cellules cancéreuses dans les capillaires de tissus est une étape importante au cours de la métastase, qui implique des interactions dynamiques entre le milieu extracellulaire et les composants intracellulaires. Des études *in vitro* sur ces processus biologiques importants nécessitent des plateformes de culture cellulaires

qui soient capables d'imiter les complexités de l'environnement extracellulaire, et notamment les géométries confinées que les cellules cancéreuses rencontrent pendant la transmigration.

La section § 2.3 porte sur la neurobiologie ; elle se concentre principalement sur le développement des cellules nerveuses et les méthodes utilisées dans la recherche. Les molécules de signalisation extracellulaire localisées à proximité des pointes des neurites en croissance (axones et dendrites) sont importantes pour le développement des neurones individuels pour former des réseaux neuronaux fonctionnels *in vivo*. Les signaux peuvent être détectés par des cônes de croissance neuronaux et ensuite déclencher une cascade d'événements moléculaires intracellulaires qui finalement affectent la croissance des neurites en cours de développement. Le mécanisme sous-jacent de ces processus biologiques importants n’est pas bien compris du fait que les plateformes traditionnelles de culture de neurones n'ont pas la capacité de présenter des signaux moléculaires multiples de manière très localisée et systématique.

Les caractéristiques uniques de dispositifs de culture cellulaire microfabriqués rendent possibles de mimer l'environnement extracellulaire complexe rencontré par le les cellules cancéreuses et neuronales au cours de leur croissance et de leur développement. Des problèmes de recherche existants dans ces domaines pourraient être résolus avec les micro-instruments nouvellement développés.

Chapitre 3 Puce microfluidique de culture de cellules cancéreuses pour l'étude de la transmigration et la métastase

Dans ce chapitre, une puce microfluidique pour l'étude de la transmigration des cellules cancéreuses est présentée. Tout d'abord, la conception et les principes de fonctionnement de la puce sont démontrés en section § 3.1. Le dispositif microfluidique à base de PDMS est constitué de deux chambres réservoirs reliées par un réseau de microcanaux. Les micro-canaux sont conçus pour imiter les petites lacunes dans la matrice extracellulaire et dans le syncytium épithélial, rencontrés par les cellules cancéreuses lors de leur transmigration. Les microcanaux très fins peuvent créer une grande résistance hydrodynamique et limiter l'écoulement du liquide. Lorsque une diffusion se produit afin de tamponner la différence de concentrations entre les deux chambres, le gradient de concentration peut être établi dans les microcanaux et attirer les cellules en vue de leur migration à travers ces canaux. Les dimensions des constrictions des microcanaux a également causé des déformations des cellules migrantes. Les qualités optiques transparentes du micro-instrument permettent une bonne observation de la déformation et de la migration des cellules dans les capillaires artificiels constitués par ces microcanaux.

Le déroulement du processus de fabrication du microsystème ainsi que les protocoles pour les expériences de transmigration sont ensuite détaillés dans la section § 3.2, y compris le processus de lithographie douce en deux étapes et le procédé de culture *in vitro* de cellules cancéreuses dans le dispositif microfluidique. Les résultats expérimentaux sont ensuite présentés en section § 3.3. Les influences de confinement géométrique sur les profils de migration cellulaire sont étudiées à l'aide de micro-canaux

de différentes dimensions. Les résultats ont montré que les déformations du noyau cellulaire rigide constituaient une des étapes les plus fastidieuses lors du processus de transmigration. Les constrictions physiques ont pour effet non seulement de changer la morphologie des cellules suite à la transmigration, mais elles affectent également de manière significative leurs profils de migration. Les déformations cellulaires drastiques dans des microcanaux étroits réduit de façon significative les vitesses de migration cellulaire et le nombre total de cellules traversant les microcanaux. Cette réduction ne peut pas être compensée par l'augmentation de la quantité de chimio-attractif qui fournissent des signaux directionnels ainsi que la force motrice pour la migration des cellules. Ceci suggère que la contrainte mécanique a non seulement pour effet d'augmenter les frictions entre les cellules et les parois du canal, mais aussi d'affecter les moteurs moléculaires intracellulaires responsables de la migration cellulaire.

Les capacités de transmigration de deux types de cellules cancéreuses, l'une hautement métastatique, MDA-MB-231, et l'autre faiblement métastatiques, MCF7, ont été comparées sur la puce microfluidique. Les résultats ont montré que des cellules MDA-MB-231 cellules pouvaient se déformer et migrer plus facilement que MCF7 dans les microcanaux de constriction, ce qui suggère que la capacité de déformation des cellules cancéreuses est étroitement liée à leurs capacités métastatiques, propriété qui pourrait être utilisée en tant que marqueur pronostique potentiel pour le diagnostic du cancer.

Chapitre 4 Déformation nucléaire et condensation de la chromatine de cellules cancéreuses transmigrées

Les fibres de chromatine occupent un volume important de noyau de la cellule. L'état de condensation de chromatine est un facteur déterminant pour les propriétés mécaniques du noyau de la cellule, cet état peut changer de façon dynamique en réponse à des stimuli extracellulaires par une modification biochimique de leurs composants moléculaires (ADN et protéines histones). Dans le chapitre 4, une série d'expériences a été effectuée pour étudier si la contrainte mécanique imposée sur les cellules cancéreuses par la géométrie confinée pendant la transmigration pouvait induire une modification biochimique sur leurs protéines histones nucléaires et ensuite affecter d'autres propriétés biologiques des cellules cancéreuses.

Dans la section § 4.1, la chromatine nucléaire des cellules cancéreuses a été colorée avec un marqueur fluorescent DAPI, les résultats ont montré que la transmigration des cellules cancéreuses a induit la condensation de la chromatine dans leur noyau, ce qui pourrait être provoqué par les contraintes mécaniques imposées sur les cellules par les constrictions géométriques dans les microcanaux.

Dans la section § 4.2, les protéines histones dans le noyau de la cellule cancéreuse ont été immuno-colorées avec différents anticorps marqués par fluorescence. Les résultats ont montré que la transmigration des cellules cancéreuses avait induit des modifications biochimiques sur leurs protéines histones ; plus d’histones méthylés et moins d’histones acétylés ont été détectés dans le noyau des cellules cancéreuses transmigrées.

Dans la section § 4.3, les cellules cancéreuses transmigrées ont été isolées à partir des puces microfluidiques et sous-cultivés dans d'autres dispositifs de culture cellulaire.

Les cellules filles récoltées à partir de cette propagation ont été analysées ; les résultats ont montré que la condensation de la chromatine et la modification des histones induite par la transmigration des cellules cancéreuses pourraient être héritées et conservées dans les cellules pendant une certaine période de temps au cours de cycles répétés de la division cellulaire.

Dans la section § 4.4, les cellules cancéreuses sous-cultivées ont été rechargées dans des puces microfluidiques pour tester leurs capacités de transmigration. Les résultats ont montré que les cellules sous-cultivées avaient des capacités de transmigration plus élevées que les cellules cancéreuses normales, ce qui pourrait être lié à la condensation de la chromatine et l'état de modification d'histone dans leur noyau.

Dans la section § 4.5, des approches pharmacologiques pour inhiber la transmigration des cellules cancéreuses dans les microcanaux ont été explorées. Les résultats ont montré que des médicaments modulateurs d'histone (MTA) inhibaient la transmigration des cellules cancéreuses dans les microcanaux de façon dépendante de la concentration, ce qui pourrait avoir des implications dans la conception d'un nouveau traitement anti-cancer.

Grâce à une série d'expériences, il a été prouvé que les déformations physiques provoquées par un stress mécanique issu des géométries confinées des microcanaux, peuvaient modifier les propriétés chimiques et biologiques des cellules cancéreuses. La combinaison des puces microfluidiques avec des méthodes d'analyse biochimique offre une bonne plate-forme pour étudier comment la signalisation de cheminement intracellulaire répond aux stimuli du microenvironnement extracellulaire.

Chapitre 5 Puce microfluidique intégrée de culture de neurones pour l'étude des trajets de développement axonal

Afin d'étudier l'effet conjoint des deux signaux moléculaires dissous et liés au substrat sur l'orientation de la croissance axonale de neurones, nous présentons dans ce chapitre, une puce microfluidique intégrée de culture de neurones incorporant des micro-motifs imprimés de protéines selon des formes géométriques contrôlées.

Tout d'abord, la conception et les principes de fonctionnement de la puce microfluidique sont démontrés au § 5.1. La puce comporte deux grands volets, un compartiment de culture cellulaire en PDMS est composé de trois micro-chambres interconnectées via des réseaux de microcanaux. La dimension des micro-canaux permet de garder le corps cellulaire du neurone (soma) dans une chambre de culture pendant que les axones peuvent se développer à travers les autres chambres. En outre, les micro-canaux empêchent les liquides de pénétrer dans des chambres adjacentes en raison de la résistance hydrodynamique élevée. Lorsque des perfusions sont mises en place pour empêcher la diffusion de substances chimiques dissoutes entre les chambres de culture, traitement chimique localisé peut être réalisé soit sur des somas ou des axones des neurones en culture. L'autre composante majeure de la puce est le substrat en verre imprimé de motifs pour l'orientation de la croissance des axones neuronaux. Des biomolécules spécifiques sont immobilisés sur le verre selon des formes géométriques définies, réalisées au moyen du procédé d'impression par micro-contact. Lorsque les neurones étendent leurs axones de la chambre de soma à la chambre de l'axone, ils peuvent réagir aux biomolécules immobilisées et ajuster le tracé de la croissance en fonction de l'information fournie par la géométrie des motifs spécifiques.

Les procédures expérimentales ont été détaillées au § 5.2, y compris le processus de lithographie douce pour la fabrication de puces micro-fluidique de culture cellulaire, les méthodes d'impression par micro-contact d'un motif de protéines sur le substrat de verre, et les protocoles pour la culture *in vitro* d'embryons de neurones corticaux de souris. Les résultats expérimentaux sont présentés au § 5.3. Tout d'abord, pour assurer la croissance et le développement des neurones sains dans le dispositif de culture cellulaire micro-usiné, une série de travaux d'optimisation a été réalisée dans le § 5.3.1 afin d'améliorer les conditions micro-environnementales dans le dispositif, y compris les revêtements de substrat de verre et le prétraitement du matériau PDMS. Au § 5.3.2, des cultures de neurones compartimentées ont été démontrées, les somas et les axones des neurones mis en culture dans le dispositif peuvent être polarisés dans les différents compartiments sur la puce et restent isolés de manière fluidique sur une longue période. L'effet de l'orientation des motifs de protéines immobilisées sur la croissance des axones des neurones a été démontré au § 5.3.3, les résultats ont montré que l'extension des axones neuronaux peut être orientée par des lignes de laminines micro-imprimées. En revanche, les axones qui rencontrent des lignes de collagènes de type IV n'ont pas été de guidés de la même manière. Le mécanisme moléculaire derrière la croissance de l'axone orienté a également été examiné au § 5.3.4 par un traitement médicamenteux localisé ; l'ajout d'un inhibiteur de la synthèse des protéines dans les chambres de l'axone, modifie de manière significative les trajets de croissance d'axones neuronaux sur les lignes de la laminine imprimées, suggérant que ces processus sont étroitement associés à des protéines synthétisées localement dans les extrémités des axones en croissance.

La puce microfluidique intégrée de culture de neurones permet l'étude de la

signalisation intracellulaire sur une base localisée et sera utile dans de nombreux domaines de la recherche en biologie cellulaire. La puce microfluidique devrait permettre l'exploration de l'effet conjoint de signaux moléculaires liés à la matrice et de signaux moléculaires solubilisés, sur le développement de la morphologie cellulaire. Elle devrait également permettre des avancées dans la fonction et la réparation tissulaire.

Chapitre 6 Conclusions et recommandations

Les capacités des instruments microfabriqués de culture cellulaire, imitant le microenvironnement compliqué cellulaire *in vivo* sont démontrées par deux nouveaux dispositifs de recherche sur le cancer et les neurosciences. Les deux nouveaux dispositifs de culture cellulaire utilisent différentes fonctionnalités des microsystèmes à des fins de recherche en biologie. Pour ce qui est de la puce microfluidique de transmigration, il a tiré avantage des dimensions de la microstructure, qui sont comparables aux dimensions des capillaires du tissu et celles des cellules cancéreuses individuelles. Par conséquent, il est apparu tout à fait approprié à l'étude de l'isolement des cellules et de leur déformation au cours du processus de transmigration ainsi émulé.

La puce microfluidique intégrée de culture de neurones utilise principalement les capacités des microstructures dans l'isolement du fluide d'une part, pour la culture cellulaire compartimentée de neurones, ainsi que les capacités d'impression de micro-motifs sur le substrat d'autre part, pour l'orientation de la croissance neuronale. Les recherches effectuées sur ces puces nouvellement développés conduiront à une compréhension plus profonde de nombreux processus biologiques importants et de meilleurs traitements médicaux des maladies qui y sont liées.

Bien que des résultats prometteurs aient été obtenus, de nouveaux développements peuvent être réalisés sur les dispositifs pour améliorer la variété, la précision et l'efficacité des expériences. Les dispositifs microfluidiques de transmigration peuvent être améliorés en incorporant des géométries différentes, tels que les angles et les courbes dans les conceptions actuelles, et en remplissant les micro-canaux avec des hydrogels avec des macromolécules dissoutes à partir de la matrice extracellulaire. Pendant ce temps l'ADN, l'ARN et les protéines peuvent également être extraits des cellules qui migrent sur les microdispositifs, en vue de l'analyse de leurs profils de transcription des gènes. La puce microfluidique intégrée de culture de neurones peut être améliorée afin d'y présenter différents types d'indices environnementaux (par exemple des stimuli mécaniques) pour les cônes de croissance des neurones, tandis que la co-culture de différent groupes de neurones peut être mis en place sur l'instrument afin d'y étudier la transduction du signal dans les réseaux de neurones ainsi définis.

AUTHOR'S PUBLICATIONS

Journal Papers

1. Y. Fu, L. K. Chin, T. Bourouina, A. Q. Liu and A. M. J. VanDongen, "Nuclear deformation during breast cancer cell transmigration," **Lab on a Chip**, Vol. 12, No. 19, pp. 3774-3778, 2012.
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