Chapter Seventeen

Microfluidic Modeling of Cancer Metastasis

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17.1 INTRODUCTION

The majority of solid tumor cancers such as breast, colon or prostate can be treated successfully through surgical resection of the primary tumor with more than 90% of the patients enjoying a long-term survival rate. However, survival rates decrease once invasion of cancer cells into surrounding local tissue, such as lymph nodes, occurs. The survival rates further decrease if the cancer is found in distal sites in the body such as lung, liver or bone [1]. Once this happens, the cancer becomes metastatic.

The metastatic process involves multiple stages. Cancer cells have to first degrade the extracellular environment and invade through the matrix surrounding the primary tumor. They further need to intravasate and survive in the circulatory system as circulating tumor cells (CTCs). Once they encounter a suitable environment for colonization such as the liver, lung or bone, they extravasate from the circulation and form metastases (Figure 17.1). The diagnosis of metastases today depend

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largely on imaging technology, for example, x-ray, magnetic resonance imaging (MRI) or positron emission tomography (PET), to name a few [2]. These imaging techniques, however, are limited by their specificity and sensitivity of detection.

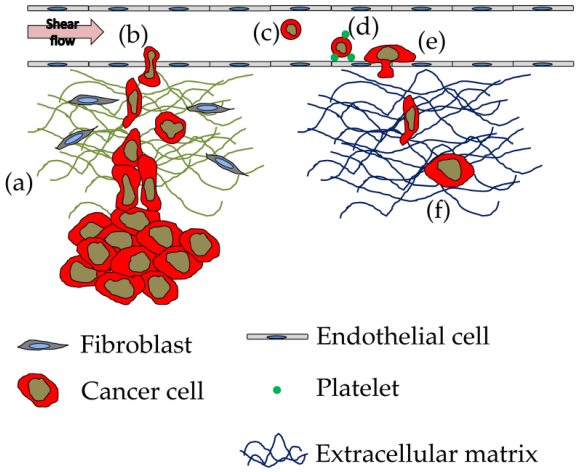

Figure 17.1. Schematic of the metastatic cascade. (a) Invasion of cancer cells through the extracellular matrix. (b) Intravasation of cancer cells into the circulatory system. (c) Circulating tumor cells (CTCs). (d) Platelet aggregation promotes adhesion of CTCs to endothelium. (e) Extravasation of cancer cells into distal sites. (f) Cancer cells colonize distal sites, forming metastases.

Microfluidics, the manipulation of fluid at the microscale, has emerged as a new and useful technology for biological research and clinical study [3]. Microfluidic devices and systems are commonly made through soft-lithography of polydimethylsiloxane (PDMS), a polymer that offers optical transparency, gas permeability, ease-of-use and biocompatibility [4]. PDMS-based microfluidic devices have demonstrated numerous benefits for biological and biomedical applications, such as cost effectiveness, high-throughput automated operation, high spatiotemporal resolution [5], low consumption of biochemical reagents [6], integration capability with three-dimensional (3D) extracellular matrix (ECM) scaffold [7] and coculture system [8], proteomic analysis at a single-cell resolution [9], to just name a few.

A significant challenge facing cancer research is determining if and when the cancer in the patient will start to invade. Successful understanding and modeling of the metastatic process will enable more effective strategies to combat it. In this chapter, we will briefly describe some of the crucial steps of metastasis: invasion, intravasation into the circulatory system and finally colonization of distal sites. We will further describe the conventional methods used to study these steps in the metastatic cascade, their limitations and how the recent development in microfluidic modeling of cancer is facilitating a better understanding of the metastatic process.

17.2 THE METASTATIC PROCESS

17.2.1 *Invasion*

Invasion is one of the first steps in metastasis. Cancer cells acquire genetic mutations or changes to its epigenetic landscape that trigger the invasion process. These changes to the genetic or epigenetic status in cancer cells can have a direct effect on the intrinsic ability of the cancer cell to invade as well as indirectly on the surrounding stromal cells to produce extracellular matrix (ECM) proteins and growth factors such as TGF-β that promote cancer cell invasion [10]. Once cancer cells have acquired the necessary mutations, they can break from the primary tumor site invading as single cells or collectively and via a variety of different methods. One widely studied process during cancer invasion is the epithelial–mesenchymal transition (EMT), where cancer cells start to express genes that enable them to transition from an epithelial origin to appear and behave more like a mesenchymal cell. As a result of this phenotypic transition, cancer cells secrete enzymes such as matrix metalloproteases that remodel the surrounding ECM and facilitate invasion [10]. Cancer cells can also maneuver their way through the matrix without degrading it; during this process, they adopt an amoeboid phenotype and squeeze through the matrix instead [11].

The physical properties of both cancer cells and ECM can change during cancer progression. While these changes are an effect of acquired genetic or epigenetic abnormalities, they play an important role in affecting cancer progression including invasion. For one, stiffening of the

ECM is commonly observed in cancer and is associated with promoting tumor initiation and invasion [12-14]. Interestingly, greater cell deformability is a general trait of metastatic cancer cells [15-17]. Other physical properties of the ECM relevant to cancer invasion include interstitial pressure. Solid tumors typically have increased interstitial pressure. While it is thought increased interstitial pressure hinders chemotherapy by posing as an obstacle to effective drug delivery, there is also recent data suggesting that interstitial pressure can regulate invasion [18, 19].

17.2.2 *Circulating Tumor Cells*

Cancer cells tend to invade along a chemical gradient, typically towards the circulatory system where a richer source of nutrient exists. Intravasation into the circulation system can occur through either newly formed or existing capillaries [20, 21]. Once in the circulatory system, circulating tumor cells (CTCs) must survive a variety of environmental factors such as anoikis (cell death induced by loss of attachment) and shear flow exerted by the circulation, as well as evade the immune system [22, 23]. Furthermore, coagulation with platelets can occur on CTCs that may result in the cancer cells being trapped and dying in micro-capillaries. At the same time, there is also evidence suggesting that such interactions between CTCs and platelets may actually facilitate the metastatic process [24]. Despite the many obstacles facing the survival of CTCs and how different they behave in the circulation, their presence in patients has been suggested to be a good prognostic indicator of patient survival in several types of cancer and is an important area of study [25, 26].

17.2.3 *Colonization of Distal Sites*

To successfully colonize a distal site in the body, CTCs must first find a suitable location that is conducive for proliferation. The most common sites of metastasis, apart from the lymph nodes are the liver, lung and bone [27]. This is facilitated by the presence of chemogradients that attract cancer cells to these sites [28]. Before invading and colonizing secondary sites, CTCs must first extravasate from the circulation. As mentioned previously, CTCs come into contact with coagulation factors

while in circulation and may interact with platelets. This CTC-platelet aggregate may facilitate binding with selectins found on the endothelium that help arrest CTCs [24]. The tumor cells can then extravasate through remodeling endothelial cell-cell junctions [29].

Once tumor cells have successfully infiltrated the secondary site, it must be able to survive within this foreign environment. It is believed that the ability of cancer cells to adapt to a new environment is limited and a majority of tumor cells die before establishing a colony [30, 31]. During this initial stage of colonization, tumors cells may remain in a state of dormancy for several years. They remain as micro lesions known as micrometastases and cannot be detected or treated with conventional methods [32, 33]. Metastatic lesions may survive as solitary cells or as a small mass through passive diffusion of nutrients. To form larger lesions, cancer cells have to ensure sufficient nutrients are present to support their growth. They do so by inducing angiogenesis, the formation of new blood vessels [34], creating a tumor vasculature that allows for increased tumor burden to be supported. The ECM in the secondary site most likely plays an important role in determining whether a metastatic lesion proliferates or remains dormant. Under the right conditions, interactions between the cancer cell and local ECM can induce dormancy [35]. Conversely, under favorable conditions, these metastatic lesions may start to proliferate [36].

17.2.4 *Conventional Technologies to Study Cancer Metastasis*

The traditional method of characterizing invasion potential of cancer cells is done using the Boyden chamber invasion assay, which is performed *ex vivo* and involves a transwell insert with a porous membrane coated with ECM proteins on it. Cancer cells are seeded onto the insert and placed into another well containing a chemoattractant. In the presence of a chemogradient, cancer cells will actively invade through the ECM protein coated porous membrane of the transwell to the bottom well. The number of cells that successfully invade into the bottom well is used as a measure of invasion potential. While the Boyden chamber invasion assay is efficient at determining how invasive a cancer cell is, visualizing the kinetics of the invasion process is difficult. Further, it is not possible to study invasion in the presence of the

microenvironmental parameters discussed above such as interstitial pressure using the Boyden chamber.

An alternative to the Boyden chamber is through the use of a 3D matrix composed of extracellular proteins, such as collagen. Typically, cancer cells are grown within a collagen gel and their invasive properties can be observed by following their migration through the matrix. The EMT process has been well studied using this method [11]. However, a drawback is that it is hard to establish invasion in the presence of a chemogradient within a collagen gel that has polymerized in a tissue culture dish.

Histological analysis of the tumor can also be used to study cancer invasion. The tumor and surrounding tissue are removed through biopsy, fixed and embedded, typically in paraffin. After embedding, slices of the fixed tumor or tissue are obtained to perform histological analysis. A major drawback of this procedure is that cells are not viable, making studying the dynamic invasion process or downstream analyses involving live cells impossible.

Intravital tumor imaging is an alternative way of studying live cancer cell invasion kinetics in an *in vivo* setting. This method typically involves generating a xenograft tumor in an animal. The animal is restrained and kept alive while the tumor exposed for monitoring through the use of a microscope. The mode of detection used in intravital tumor imaging is typically based on fluorescence or bioluminescence. The advantage of this method is that the tumor is grown in an *in vivo* setting similar to its native environment and the dynamic invasion process can be studied over time. However, this technique is costly to perform and difficult to execute [2].

There are several platforms currently available that are used for capture and studying CTCs from blood specimens [37]. They mainly involve antibody based approaches targeting markers of epithelial cells to separate CTCs from non-tumor cells of hematological origin [26, 38]. A drawback to using such method is that CTCs are poorly characterized and known to be heterogeneous. Further, during the metastatic process, cancer cells may undergo EMT and lose their epithelial markers and express a totally different set of genes [39].

Currently, detecting metastases is done mainly using imaging technologies, which require imaging agents to be taken up by cancer cells before detection. The challenges lie in designing molecules that will

be specifically taken up by cancer cells and at the same time compatible with the mode of detection. An example of the most successful and widely used imaging agent is 2-deoxy-2-(18F)fluoro-D-glucose (FDG), a glucose analogue. As improvements to both imaging technologies as well as imaging agents continue, we can expect increased sensitivity in future imaging tests as well as better specificity towards tumor cells.

The current tools used have played an instrumental role in furthering the understanding of metastasis. However, each system has their limitations that prevent a deeper understanding of metastasis as a multistep dynamic process. But, with advancement in technology, these limitations are slowly removed. In recent years, the field of microfluidics especially has been stepping up as a major player in helping to improve our understanding of metastasis.

17.3 MICROFLUIDICS FOR STUDYING CANCER METASTASIS

While the use of microfluidics in studying cancer metastasis is extensive, it is worth noting that two typical chip configurations, as shown in Figure 17.2, have been most commonly used. The first configuration contains three microchannels in which the central and the side channels are connected by an array of blocks forming fluidic constrictions or barriers [40, 41]. Owing to dominant surface tension force, the block barrier serves as a cage to confine the biological matrix solution in the central channel and prevent the solution from flowing into the side microchannels. As the matrix can be embedded and gelated with cells, drugs, or other biological ingredients for cell immobilization and localization, this configuration is suitable to study cancer invasion, intravasation and extravasation, and the epithelial–mesenchymal transition (EMT) (see detailed discussion in Section 2). In the second configuration, two main microchannels are connected with interconnecting microchannels, which are typically as thin as 3 μm to minimize convection flow between the two microchannels [42, 43]. This configuration is often used to study directional cell migration such as chemotaxis and cell deformation through the interconnecting microchannels under a chemical concentration gradient.

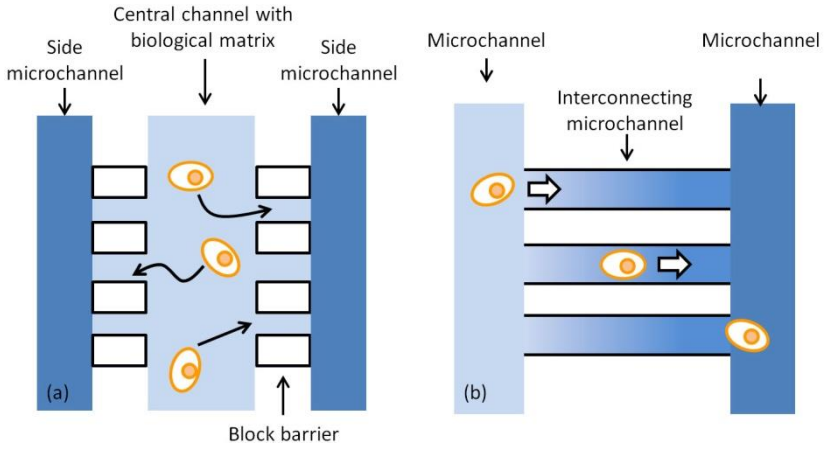

Figure 17.2. Typical microfluidic chip configurations for modeling metastasis. (a) A central microchannel has a cage-like structure to confine the shape of the biological matrix. (b) Interconnecting microchannels confine cell migration between two main microchannels.

17.3.1 *Microfluidic Modeling of Epithelial–Mesenchymal Transition (EMT)*

Combining the capability of generating 3D microenvironment and requiring only small amounts of chemicals, a recent microfluidic study by Aref *et al.* using lung adenocarcinoma A549 spheroids, cancer cells that can revert from an intermediate mesenchymal-like phenotype to an epithelial-like phenotype, demonstrated that microfluidics could offer a power approach for therapeutic drug screening for EMT [44]. In this study, A549 spheroids suspended in collagen I hydrogel were first seeded in the central microchannel using an array of fluidic constrictions and surface tension effect as shown in Figure 17.2 (a). Human umbilical vein endothelial cells (HUVECs) were subsequently loaded to the two side channels to form a HUVEC monolayer along the gel surface. Except for one of the 13 drugs tested using this microfluidic platform, significantly lower the half maximal inhibitory concentration (IC50) doses necessary to inhibit EMT was observed for spheroid maintained in the 3D microenvironment as compared to carcinoma cells in isolation in conventional 2-dimensional (2D) microwell systems, underscoring the

significant difference of cancer cells in response to drug treatments between 2D and 3D, and between monoculture and co-culture systems.

17.3.2 *Microfluidics for Capture and Informative Analysis of Circulating Tumor Cells (CTCs)*

Microfluidic chips have demonstrated reliable capture of CTCs from whole blood of cancer patients. One of the pioneering devices, reported by Nagrath *et al.*, involved flowing patient blood onto a microfabricated flow chamber containing an array of pillars conjugated with antibodies against surface markers of cancer cells [45]. Since then, numerous creative concepts using microfluidics have been proposed to improve capture efficiency and purity of CTCs directly from blood specimens by taking advantage of the differences in biophysical and surface properties between cancer and non-cancer cells. One example involved capturing CTCs using nanoscale rough surfaces etched on glass slides. This technique is advantageous as it does not require antibodies to capture the CTCs and makes use of the intrinsic preference of CTCs for adhesion on rough surfaces over smooth ones [46]. Another example involved the generation of a spiral shaped microfluidic channel. As blood was continuously passed through the microfluidic channel, differential centrifugal forces were exerted on the blood cells and CTCs that allowed the cells to separate based on size [47]. A third example combined microfluidic chaotic mixing using herringbone structures with silicon nanopillar surfaces coated with anti-EpCAM antibodies (Figure 17.3) [48]. Owing to enhanced cell-surface interactions as well as increased cell capture surface area, superb capture efficiency for CTCs was reported by Wang *et al.* (> 95%).

Microfluidics has also been applied as model systems to study adhesion of CTCs [49]. For example, Zhen *et al.* proposed a simplified biophysical model to study the effects of cell receptor and surface ligand density on dynamic states of adhesion of CTCs on a microfluidic channel functionalized with capture antibodies. Their biophysical model was based on a receptor-coated sphere moving above a solid surface immobilized with capture ligands. The mathematical analysis and modeling for capture of CTCs were based on calculation and numerical simulation of Langevin equation and an empirical formula with receptor-ligand bonds modeled as linear springs separated by a gap. The

authors also examined two breast cancer cell lines, MDA-MB-231 and BT-20, both expressing EpCAM, in microfluidic channels coated with anti-EpCAM or anti-N-cadherin antibodies. Besides three dynamic states (firm adhesion, rolling adhesion, and free motion) CTCs going through as verified by experiments, simulation and analysis, Zhen *et al.* were able to estimate the cell-surface gap and spring constant properly. Importantly, all measured and simulated results could be generalized as an exponential correlation between the CTC capture ratio and the normalized flow rate.

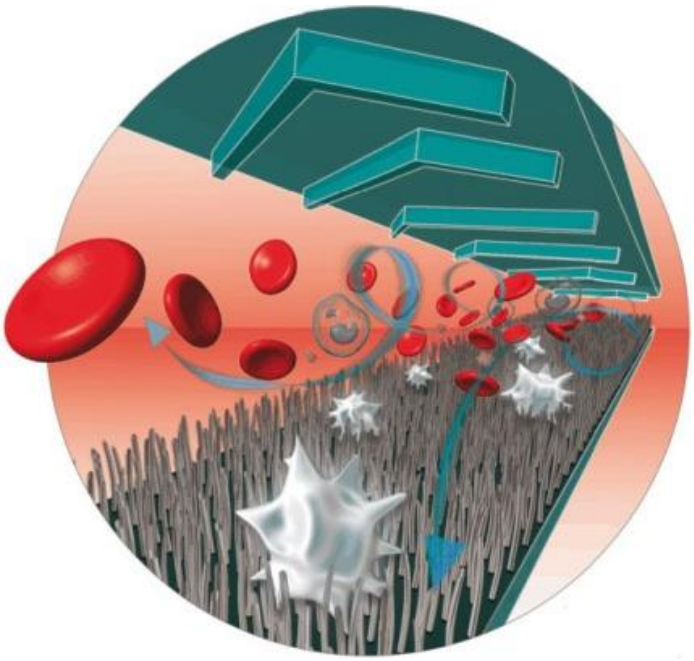

Figure 17.3. Functionalized nanostructured substrate combined with a microfluidic chaotic mixer to capture circulating tumor cells with high efficiency. Adapted from [48]. Reprinted with permission from John Wiley and Sons.

Similarly, Song *et al.* performed an interesting comparative experiment on tumor cell adhesion modulated by endothelium [50]. After a confluent monolayer of human dermal microvascular endothelial

cells (HDMECs) was cultured on top of a semi-porous polyester membrane sandwiched between top and bottom PDMS microchannels, the authors found that twice as many breast cancer cells could adhere to the endothelium when HDMECs were treated with CXCL12 basally compared to apically. Such result suggests that the orientation or polarity of the endothelium can be critical in regulating vascular transport and arrest and retention of CTCs.

In addition to studying the physical properties of CTCs, microfluidics has also contributed towards a better understanding of CTC biology. By coupling the use of a herringbone microfluidic chip with antibodies into a single platform, it was possible to isolate and characterize CTCs from breast cancer patients [51]. From this study, the authors found that EMT markers were enriched within the isolated CTCs as compared to cancer cells within primary tumor, reinforcing the clinical importance of EMT as a key player in the metastatic process. In a separate study by Ameri *et al.*, the authors generated a xenograft model of human breast cancer in mice and used a magnetic based microfluidic device to isolate CTCs generated from these xenograft models. Briefly, magnetic beads containing antibodies recognizing EpCAM were added to blood harvested from mice and the labeled blood was passed through the microfluidic device that allowed automated recovery of CTCs. The authors were further able to demonstrate that isolated CTCs behaved more aggressively than the cells from the primary tumor in response to hypoxia and established a relationship between hypoxia and CTCs [52].

The use of microfluidics has also helped improve on monitoring clinical progression of cancer. In particular, Maheswaran *et al.* isolated circulating tumor cells from lung cancer patients using a microfluidic device and analyzed these CTCs for EGFR mutations. The authors found mutated EGFR in CTCs isolated from patients that underwent tyrosine kinase inhibitor therapy. These mutations conferred resistance to tyrosine kinase inhibitors. Furthermore they studied CTCs isolated from pre-treatment patients and observed a negative correlation between pre-existing mutated EGFR in CTCs from pre-treatment patients and survival. This work demonstrates the capability of using microfluidics in improving clinical prognosis and perhaps even predict therapy outcome [53].

17.3.3 *Microfluidics to Study Cancer Cell Migration*

By incorporating a microfluidic gradient generator to produce flows with laterally uniform, linear, polynomial or complex concentration gradients of soluble molecules including epidermal growth factor (EGF), anti-EGF and CXCL12 ligands, researchers studied human metastatic breast cancer cell line MDA-MB-231 in terms of cell motility, speed and directionality [54-56]. Results illustrated that chemotaxis of metastatic cancer cells could depend on the shape of chemical gradient profile as well as the chemical concentration range.

Integrating microfluidics with modern microscopy technologies allows for real-time observation of tumor cell migration in geometrically confined environment, which is difficult in conventional assays. Two research groups applied microfluidic channel structures shown in Figure 17.2 (b) to emulate migration of brain cancer stem cells through interstitial spaces and that of breast cancer cells by the influence of nuclear deformation through endothelial-lined capillaries [57, 58]. In both cases, the interconnecting microfluidic channels with their sizes ranging from 3-5 μm were assembled by either reversible or irreversible PDMS bonding against glass slides. The microfluidic chips were further coated with poly-L-lysine or fibronectin, to enhance cell attachment. Time-lapse imaging was carried out to capture real-time dynamics of cell migration along the interconnecting channels for a period of 2 days.

With a more sophisticated fabrication to embed micro-valves in microfluidic chips, researchers were able to use phase-contrast microscopic time-lapse images to detail effects of cell-cell interactions on cell migration through paracrine signaling [57]. A simple microfluidic patterning technique was also recently reported by Wang *et al.* that could facilitate screening of potential anti-migratory agents, beneficial for drug discovery compared to conventional wound-healing assay [59]. Specifically, after three cancer cell lines with different metastatic potentials were individually plated and confined inside microchannels of a PDMS stamp, the stamp was removed and free movements of monolayers of cancer cells were imaged over time. Migration rate of cancer cells under the treatment of two anticancer drugs, curcumin and apigenin, was successfully evaluated using this microfluidic patterning technique.

17.3.4 *Microfluidic Tools to Study Interactions of Cancer Invasion, Intravasation, and Extravasation with the Microenvironment*

Several research groups [7, 40, 41, 60] studied invasion of tumor cells through endothelium and/or ECM using the two microfluidic configurations shown in Figure 17.2. The essential elements in such microfluidic cancer metastasis models are the ECM, commonly made of collagen, Matrigel and agarose, as well as the endothelial monolayer, commonly using HUVECs. By exploiting the dominant effect of surface tension, ECM prepolymers can be localized and polymerized inside interconnecting microchannels or micro-cages in microfluidic chips. These gelated ECM media are porous to allow processes like chemotaxis and immunostaining by diffusion or convection of chemoattractants and biomolecules.

Using microfluidic cancer metastasis models with gelated ECM media, researchers successfully demonstrated sustained maintenance of concentration profiles of soluble factors for a prolonged period of time by simply connecting a microchannel made in ECM with a small source and a large sink [61]. Periodically adding factors and replenishing the sink by an operator, the microchip could generate pseudo-steady linear as well as non-linear concentration gradients up to 10 days. Such microfluidic tools were successfully used to study invasion of metastatic rat mammary adenocarcinoma cells (MtLN3) into surrounding matrix.

Recently, Shin *et al.* reported a microfluidic chip containing serially connected chambers and external screw valves that could be used to study both cancer intravasation and extravasation simultaneously [62]. In the intravasation chamber, colon cancer cells, either metastatic LOVO or non-metastatic SW480, were embedded in polymerized Matrigel. The extravasation chamber was coated in sequence with poly-L-lysine, fibronectin and HUVECs. The two chambers were flown with media with shear stress of a physiological range (1-5 dyne·cm^{-2}). By counting the number of cancer cells escaped from the intravasation chamber and arrested onto the extravasation chamber, the ability of cancer cells to intravasate and extravasate under different drug treatment conditions were characterized by the authors.

17.3.5 *Microfluidic Study of Cancer Cell Deformation*

Mobility of cancer cells are connected to the physical and mechanical properties of the cells and the surrounding microenvironment such as cell size and deformability, ECM porosity and deformability, and blood vessel size and pressure. Recently, different microfluidic cell deformability assays have been successfully developed to allow single cancer cells to flow or migrate through confining structures such as microscale orifices and channels. These cell deformability assays have been proven useful to study (i) how cancer cells traverse through blood vessel during metastasis [63, 64], (ii) difference in cell deformability between benign and malignant cancer phenotypes [65], and (iii) mechanical effects on behaviors of cancer cells through sub-nucleus physical confinement [42]. Experimental parameters such as cell entry time, transit velocity, elongation index, motility, viability, proliferation, have been commonly analyzed in such microfluidic cell deformability assays to quantify how cancer cell migration and motion are dictated by their intrinsic deformability property. In addition to microfluidic confining structures, optofluidics tools, such as optical tweezers, have been recently integrated with microfluidics to examine functional correlations between intrinsic deformability property of cancer cells and their metastatic potential [17].

17.3.6 *Microfluidic Modeling of Angiogenesis*

Instead of fabricating typical PDMS microfluidic chips, a research group constructed a plate structure formed by aggregating poly(lactic-co-glycolic acid) (PLGA) particles to resemble and study tumor angiogenesis. The plate attached with HUVECs was laid out within the hydrogel matrix, and cancer cells were either placed in the center or spread evenly to mimic the initial phase of a tumor before vascularization or a highly vascularized tumor respectively. Their engineered tumors showed greater drug resistance compared to cancer cells cultured in a traditional 3D setting [5].

17.3.7 *Microfluidics for Cancer Imaging*

Integrated microfluidic radioassays for glycolysis analysis in small tumor cell populations were recently developed by Vu *et al.* to detect and image very low activity levels of beta emitting isotope [66]. This microfluidic radioassay has achieved highly sensitive imaging of a radioactive tracer 18F-FDG uptake in small mouse melanoma cell populations down to a single-cell level (Figure 17.4). Further, by precisely controlling dynamic operations under *in situ* imaging and subsequent data modeling, this microfluidic radioassay was shown to be capable of obtaining kinetic rate constants of 18F-FDG metabolism. This microfluidic radioassay system suggested an exciting new way to quantitatively study transport and reaction of biomolecules within cancer cells at the culture scale.

Micro Image Cytometry (MIC) technology, a system composed of a microfluidic cell array chip, image acquisition and cytometry analysis, was recently developed by Sun *et al.* to study cancer cells [67]. Coupled with systems pathology analysis, the MIC technology developed by Sun *et al.* was shown to be capable of quantitative, single-cell proteomic analysis of multiple signaling molecules using only about 1,000 single cells. Using MIC, simultaneous measurements of four critical signaling proteins (EGFR, PTEN, phospho-Akt and phospho-S6) relevant to the oncogenic PI3K/Akt/mTOR signaling pathway had been achieved in individual cancer cells by Sun *et al.*, with their results showing meaningful correlations between measurements of minute patient samples and clinical prognosis.

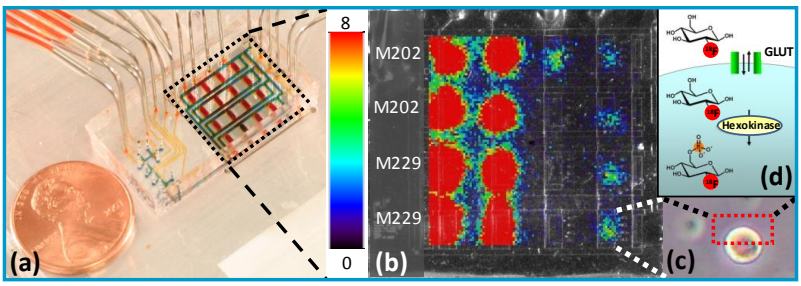

Figure 17.4. FDG uptake as a way to image cancer metabolism. (a) Schematic of the integrated microfluidic radioassay and the corresponding (b) Radioassay image. (c) Micrograph showing a single cell in a chamber. (d) Glycolysis kinetics studied using the integrated microfluidic radioassay. Adapted from [66]. Reprinted with permission from the Society of Nuclear Medicine and Molecular Imaging, Inc.

17.4 CONCLUSION AND PERSPECTIVE

In this chapter we have reviewed and highlighted the biological significance of using microfluidics to study and model cancer metastasis. While not a comprehensive review of all available microfluidic devices, the various microfluidic models discussed here have suggested microfluidics as a promising and powerful research tool for new and in-depth understanding of cancer metastasis as compared to traditional assays.

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