Microfluidics modelling of interstitial flow through a tumor and the study of tumor cell under dielectrophoresis

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2021

Tay, B. Z. (2021). Microfluidics modelling of interstitial flow through a tumor and the study of tumor cell under dielectrophoresis. Doctoral thesis, Nanyang Technological University, Singapore. <https://hdl.handle.net/10356/154459>

<https://hdl.handle.net/10356/154459>

<https://doi.org/10.32657/10356/154459>

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Microfluidics modelling of interstitial flow through a tumor and the study of tumor cell under dielectrophoresis

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NTU Institute for Health Technologies (HealthTech NTU)**

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**Interdisciplinary Graduate Programme
NTU Institute for Health Technologies (HealthTech NTU)**

A thesis submitted to the Nanyang Technological University in partial
fulfillment of the requirement for the degree of
Doctor of Philosophy

2021

Abstract

90% of cancer-related mortalities are due to the metastatic spread of cancer. Interstitial pressure and flow within the tumor microenvironment have been identified to be influential in the metastatic spread of cancer. This study focuses on biophysical effect of interstitial pressure and flow on the detachment of MCF-7 cells of varying expression levels of E-cadherins from primary tumor. The expression levels of E-cadherins affects the intercellular adhesivity of MCF-7 cells which relates to the varying degree of invasiveness in cancers. This allows for the study of heterogeneity of MCF-7 cells which closely mimics the *in vivo* tumor. To affect the expression levels of E-cadherins, MCF-7 cells are cultured in 1.5mM, 1.7mM and 1.9mM calcium concentration culture medium. MCF-7 cells cultured in different calcium concentration medium are observed and measured for size and shape differences to study the morphological differences. There are significant morphological differences. ELISA test is conducted, and the results show that the expression level of E-cadherin in MCF-7 cells cultured in 1.5mM calcium concentration culture medium is 1.46 times lower than MCF-7 cells cultured in 1.7mM calcium concentration culture medium and 2.20 times lower than MCF-7 cells cultured in 1.9mM calcium concentration culture medium. Flow is introduced to MCF-7 aggregate in a microfluidic device and pressure differences between the afferent and efferent ends are recorded at 1s time interval. Fluid flows through the porous aggregate at a slower rate than the flow rate at the afferent end causing an increase in pressure difference. Under high loading rate ($1\mu\text{l}/\text{min}^2$), for MCF-7 aggregates cultured in 1.9mM calcium concentration culture medium, which express higher E-cadherin, fracture in aggregates which mimics the detachment of the tumor cells from the primary tumor is observed at a pressure difference of 1036Pa. For MCF-7 aggregates cultured in 1.7mM and 1.5mM calcium concentration culture medium, fracture is observed at a pressure

difference of 510.2Pa and 321Pa respectively. These findings show that MCF-7 cells with higher expression of E-cadherins can withstand higher pressure buildup at the afferent ends and fractures occur and detach at a higher critical fracture pressure than MCF-7 cells with lower expression of E-cadherins. Under low loading rate ($0.5\mu\text{l}/\text{min}^2$), the mean critical fracture pressure is not affected by the change in E-cadherin expression levels. The role of extracellular matrix in the detachment of MCF-7 cells is also studied by varying the fibronectin concentration in the coating of microfluidic device. Results show that an increase in fibronectin concentration which translates to stronger cell-ECM adhesions would cause an increase in mean critical fracture pressure.

A simple DEP setup was used on MCF-7 cells. DEP induced lateral movement of MCF-7, forming chain of MCF-7 cells that are parallel to the microelectrodes. Under positive DEP, MCF-7 cells are attracted to the microelectrodes. Under negative DEP, MCF-7 cells are repelled away from the microelectrode to form a chain of cells. The cells adhere to each other. This opens up opportunity for collection of this chain of MCF-7 cells to study single cell-cell adhesion on MCF-7.

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

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I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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Assoc Prof Marcos

Authorship Attribution Statement

Acknowledgements

Throughout my pursue of PhD, there are many people who has extended their help to me in one way or the other. However, there are a few individuals who has gone above and beyond to help me, encourage me, teach me, motivate me and give me great advice.

First, I would like to thank and offer my greatest gratitude to my main supervisor, Prof Marcos (School of Mechanical and Aerospace Engineering, Nanyang Technological University NTU) for his unwavering support, guidance, advice, counselling and motivation. Prof Marcos is always there for me. Despite his busy schedule as an appointment holder in MAE, he always takes time off his schedule to answer my questions and check in on me. He also helped me find relevant expertise when there is a need. I cannot thank him enough.

Next, I would like to thank my co-supervisor, Prof Li Hoi Yeung (School of Biological Sciences, Nanyang Technological University NTU). Since this is an interdisciplinary work and the fact that I do not have much expertise in the initial phase, Prof Li provided good insights and advice to lead me on the right track.

I would also like to thank Dr Chan Wing Yue from Singapore General Hospital. He is always providing tips on conducting biological experiments. Being an alumnus of NTU himself, he knows how pursuing PhD is in NTU is and do not hesitate to give me advice and I truly thank him from the bottom of my heart.

I would also like to extend my greatest gratitude to Prof David Gonzalez-Rodrigues (University of Lorraine, Metz, France). He is another person who is always there to render help to me whenever I need them. He also gave me valuable advice. Despite the time difference between

Singapore and France and his busy schedule, he is always willing to accommodate my time to meet up with me online and advise me. Thank you so much.

I would like to thank all the research fellows and PhD students from Marcos Research Team. They include Dr Kong Tian Fook, Dr Yao Xin, Dr Shen Xinhui, Dr Tan Pei Yen and the graduated Dr Tran Duc Quang for their support and help.

To be able to complete my thesis, I also have to thank Ms Heng Cheen Hoon of NTU Biological Lab. She helps me in the lab logistics and guide me in using the equipment.

Finally, I would like to thank NTU and IGS-HealthTech for providing me with the research scholarship to fulfil my dreams. Thank you.

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1 Chapter 1 - Introduction

1.1 Background

Cancer is the leading cause of deaths worldwide. According to the World Health Organization, there were approximately 19.3 million new diagnoses of cancer and almost 10 million cancer mortalities in 2020 [1].

Closer to home, in Singapore, a total of 74,536 new cancer cases were diagnosed during the period of 2014-2018. Of these new incidence cases, 48.5% were males and 51.5% were females. From 2017-2019, cancer is the leading cause of death in Singapore standing at ~29%. That is between 6090-6271 cancer related deaths per annum from 2017-2019 [2]. The 3 most frequent cancers in males are colo-rectum (16.9%), prostate (14.8.0%) and lung cancers (14%). The 3 most frequent cancers in females are breast (29.3%), colo-rectum (13.3%) and lung cancers (7.5%). These were the findings of 2014-2018 (Figure 1-1)

Gender	Rank	Incidence			Gender	Rank	Incidence		
		Site	No.	%			Site	No.	%
Male	1	Colon & rectum	6129	16.9	Female	1	Female breast	11232	29.3
	2	Prostate	5368	14.8		2	Colon & rectum	5109	13.3
	3	Lung	5083	14.0		3	Lung	2862	7.5
	4	Liver	2758	7.6		4	Uterus	2769	7.2
	5	Lymphoid neoplasms	2358	6.5		5	Ovary	1897	4.9
	6	Non-melanoma skin	1975	5.5		6	Lymphoid neoplasms	1809	4.7
	7	Stomach	1605	4.4		7	Non-melanoma skin	1568	4.1
	8	Kidney	1494	4.1		8	Thyroid	1483	3.9
	9	Myeloid neoplasms	1232	3.4		9	Stomach	1160	3.0
	10	Pancreas	1119	3.1		10	Cervix	1088	2.8
		All sites	36187	100			All sites	38349	100

Figure 1-1 Ten most frequent cancers in Singapore from 2014-2018 for males (Left) and females (Right) [108].

Breast cancer is the most prevalent cancer in Singapore, standing at 29.2% of all females which is 9284 new incidence cases of breast cancers in the period of 2010-2014. This is followed by colo-rectum and lung cancers in both genders. Breast cancer is the most common cancer in Singapore for one gender.

Tumors are growths in our bodies, and they may be benign or malignant. When tumors are benign, the growth is localized. When tumors are malignant, they are termed cancers. Cancer as defined by the National Cancer Institute as a collection of related diseases where abnormal cells in the body begin to divide without stopping and invade into nearby tissues [3]. Cancerous cells invade into nearby tissues and affect the structures and functions of the tissues. When detected early, a malignant tumor can be surgically removed from its primary site before the cancerous cells spread to distant sites of the body. This greatly increases the patients' chance of a full recovery [4]. However, early detection of tumor is difficult. More often than not, our bodies do not show significant symptoms of tumors or growths in the body; hence it is relatively difficult for early detection. Once the early detection window is missed, cancerous cells would detach from the primary site and spread to other parts of the body and this process is termed metastasis. Treatments are less successful after metastasis is detected [5].

90% of cancer related mortalities is due to the metastatic spread of cancer [6]. Despite the high mortality rate due to the metastatic spread of cancer, minimal funds as a percentage of total cancer research funding are devoted for metastasis research. The highest funding for metastasis research as a percentage of total cancer research funding is 11.6% of the Oncosuisse, Swiss Cancer League and Foundation Cancer Research, Switzerland, while the lowest funding is 2.3% of the American Cancer Society, USA [7]. The average funding for metastasis research is around

5% of the total cancer research funding (Figure 1-2). In comparison to the 90% cancer related mortalities due to metastatic spread of cancer, the funding devoted to metastasis research is relatively minimal. More has to be done in the research of metastasis.

Organisation/country	Period	Cancer research funding	Metastasis research [*]	% Devoted to metastasis
Deutsche Krebshilfe (German Cancer Aid) ^a	2007	73,150,000 EUR	3,191,000 EUR	4.3
National Cancer Research Institute Partners, UK ^b	2002	£ 257,000,000	£ 74,000,000	2.9
National Cancer Research Institute Partners, UK ^b	2006	£ 391,000,000	£ 200,000,000	5.1
Oncosuisse, Swiss Cancer League and Foundation Cancer Research, Switzerland ^c	2006–2008	43,943,750 CHF	5,094,400 CHF	11.6
European Union (FP6) ^d	2002–2006	485,000,000 EUR	24,205,137 EUR	5.0
American Cancer Society, USA ^e	2009	485,000,000 \$	11,000,000 \$	2.3
Canadian Cancer Research, Alliance ^f	2006	390,200,000 CAD	25,200,000 CAD	6.5

Figure 1-4 Funding for metastasis research as a percentage of total cancer research funding [7].

1.2 Metastasis

Metastasis is a process where the cancerous cells detach from the primary tumor and spread to distant sites of the body. This is a complex process which involves multiple steps. The metastatic cascade starts out as a malignant tumor that has uncontrollable growth and invasive properties where the tumor invades adjacent tissues. Due to multiple biophysical and biochemical triggers, cancerous cells would detach from the primary tumor, intravasate into the blood and lymphatic circulation, and get transported through the circulatory systems to all parts of the body. These circulating tumor cells (CTCs) are arrested at distant sites, extravasate from the circulatory systems and colonize into a secondary tumor [6]. Metastatic process is highly inefficient because very few of the circulating tumor cells (CTCs) successfully colonize and start to grow at secondary sites. Despite metastasis being a highly inefficient process, cancer related mortalities are on the rise and 90% of these cancer related mortalities are due to the metastatic spread of cancers.

1.3 Project Motivation and Objective

As mentioned, 90% of cancer related mortalities are due to the metastatic spread of cancer and only around 5% of the total funds for cancer research are devoted to the study of metastasis, hence relatively little is known about metastasis. The metastatic cascade is a complex process that involves many steps. The initial phase of metastasis is the detachment of the cancer cells from the primary tumor. This detachment is influenced and affected by biophysical factors and biochemical gradients. Of the many biophysical factors, interstitial fluid pressure is highly influential in affecting the initial detachment of cancer cells from the primary tumor [8]. Interstitial pressure difference that causes the fracture and masses of cells to dislodge, kickstarting the metastatic cascade has been studied on MCF-7, a breast cancer cell line. However, the effects of interstitial fluid pressure on different intercellular adhesivity of MCF-7 to study the heterogeneity that exists in tumor is still not well studied.

In light of rapid developments and advantages of microfluidic devices, the author aims to:

1. Use microfluidic device to study interstitial fluid pressure difference that causes fractures and detachment of varying intercellular adhesivity of MCF-7 cells in a tumor,
2. Use microfluidic device to study interstitial fluid pressure difference that causes fractures and detachment with varying concentration of extracellular matrix components, and
3. Employ dielectrophoresis to sort viable MCF-7 cells from non-viable cells and visualize the interactions DEP has on MCF-7 cells

This allows for comparison between different MCF-7 adhesivity and study the effect of these variations under interstitial fluid pressure. This study is done by simulating the *in vivo*

environment of a tumor and generating a flow through a cell aggregate. The microfluidic device is coated with fibronectin which is one of the major components of the extracellular matrix. The coated fibronectin takes on the role of the extracellular matrix which acts as a scaffold for the tumor. Varying the concentration of fibronectin coating allows for the study of the effects of interstitial flow on varying cell-extracellular matrix strength. Experimental data are collected for analysis and discussion.

2 Literature Review

2.1 Cells

Cells are the building blocks of our human bodies. Cells differentiate to form specialized cells. Groups of identical cells form tissues which has specific functions. There are 4 main groups of tissues in our body. They comprise of muscle tissues, epithelial tissues, connective tissues and nervous tissues. Different tissues are functionally grouped together to form an organ system. An organ system performs a specific function. An example would be the heart. Heart is an organ that consists all 4 groups of tissues and these tissues are functionally grouped together to carry out a specific function. The human body is made up of many different organs carrying out different functions.

Figure 2-1 Cell and its organelles. The cell nucleus contains all the genetic information of a cell [100].

Each cell has multiple organelles that are crucial to its survival. Of these organelles, the cell nucleus is the most important. The cell nucleus contains chromosomes, which are made up by strands of deoxyribonucleic acid (DNA). DNA contains genes which hold genetic information of cells (Figure 2-1). These genes control the production of proteins and ribonucleic acid (RNA). Proteins and RNA control cellular activities of the cells [9] [10].

Most cells undergo reproduction through cell division and this process is known as mitosis. During mitosis, a cell organizes, duplicates DNA in the nucleus and divides into two identical cells with the same genetic information. The cell division process is strictly orchestrated by the genes of the cells hence cells reproduce only when needed and in an organized manner. However, mutations still do occur during cell division. These mutations cause changes in the genes of cells after cell division [11] [12]. The changes can include eliminating, damaging or replicating of genes in the new cells after cell division. These mutations in the genes will cause the cells to receive inaccurate information to produce proteins and RNA which in turn cause a change in the cellular activities. Some mutations cause cells to over-produce proteins, while some may cause cells to under-produce proteins and these mutations can happen at any genes. If the mutations happen in particular genes that control the production of proteins that trigger cell division or stop cell apoptosis, uncontrollable cell growth will happen [13]. This uncontrollable cell growth is the onset of cancer.

2.2 Cancer and Metastatic cascade of events

Cancers are malignant tumors that have the characteristics of uncontrollable growth, do not go through apoptosis [14] [15] and have invasive properties. Cancer starts off as a tumor or growth in our body at one site and this tumor is termed as a primary tumor. Tumor cells like normal cells require increasing blood supply and nutrients to proliferate. Angiogenesis [16] [17] recruits new blood vessels to the primary tumor and is induced by the tumor cells through the release of angiogenic factors such as Vascular Endothelial Growth Factor (VEGF) [18]. Blood and nutrients are now able to reach these tumor cells, and this greatly helps in the proliferation of tumor cells. Tumor angiogenesis is rapid and haphazard. This leads to leaky blood vessels that are hyper-permeable in the tumor [19]. Besides angiogenesis, the tumor cells also induce the formation of lymphatic vessels around the tumor. This is done through the release of lymphangiogenic factors such as Vascular Endothelial Growth Factor (VEGF-C, VEGF-D) and Transforming Growth Factor (TGF- β) by the tumor cells [19] [20].

Tumor cells detach from primary tumor and start the metastatic cascade [21] [22] due to various reasons. These reasons can be classified into 3 main categories. They include the physical factors, biochemical gradients and biological properties of cancer cells.

Firstly, physical factors that cause the detachment of tumor cells are contributed by a combination of effects from leaky blood vessels and the production and remodeling of extracellular matrix. Leaky blood vessels cause excess accumulation of fluid in the tumor. The excess fluid and blood in the tumor generate shear stress on the tumor cells and this stress activates latent TGF- β . TGF- β induces the differentiation of fibroblasts to myofibroblasts. Myofibroblasts are cells in the extracellular matrix and have critical roles in tumor proliferation

and metastasis. Myofibroblasts increase secretion of angiogenic factors, increase cytokines which are biochemical that promote tumor growth, induce the production of matrix metalloproteinases (MMPs) which helps in the dissemination of cancer cells to distant sites and has contractile properties. Myofibroblasts cause the extracellular matrix to contract [23] [24] and this is a positive feedback which activates more latent TGF- β (Figure 2-2) [8]. Extracellular matrix contraction and the accumulation of excess fluid in the tumor's interstitial space elevate interstitial fluid pressure [25]. Heightened interstitial fluid pressure leads to a steep pressure gradient at the periphery of the tumor [26]. This is a driving force for the interstitial fluid to flow out of the tumor. The interstitial flow will generate a force and cause the detachment of tumor cells to the blood and lymphatic vessels around the tumor. Elevated interstitial fluid pressure within the tumor also reduces the pressure gradient between tumor and blood vessels. Conventional chemotherapy drugs are administered intravenously. With the reduced pressure difference between blood vessels and tumor, reduced concentration of chemotherapy drugs is being delivered to the tumor. Increased interstitial fluid flow out of the tumor also prevents the convective flow of drugs within the tumor [25].

Figure 2-2 Summary of biochemical and biophysical factors influencing the migration of tumor cell. Mechanical signals include stiffness of the extracellular matrix (ECM), fibroblast generated tensions. Biochemical signals include autocrine gradient and NMPs [57]

Secondly, biochemical gradients play a crucial role in the detachment and migration of the tumor cells from the primary tumor under interstitial flow [27] [28] [29]. The biochemical being discussed here includes the CCL19 and CCL21. These are chemokines that are released by tumor cells under interstitial fluid flow. These chemokines are ligands to CCR7 which is a receptor found on the tumor cells. With fluid flowing out of the tumor, CCL19 and CCL21 are carried along with the flow to the draining lymphatic vessels. This creates a chemokine gradient. Tumor cells that express CCR7 receptors will detach and migrate towards the lymphatic vessels with a higher concentration of these ligands. This mechanism is termed autologous chemotaxis (Figure 2-3). This means that the chemokines secreted by the tumor cells are ligands to their own receptors. Autologous chemotaxis promotes the detachment and migration of tumor cells from the tumor to lymphatic vessels, which is another starting point for metastasis [29].

Figure 2-5 Pictorial description of CCR7 autologous chemotaxis [29],
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Lastly, biological properties of tumor cells also contribute to the detachment of tumor cells from the primary tumor. Cancer cells are mutated cells with deleted or additional genes. This mutated cancer cells may lose the genes to signal for proper production of surface adhesion receptors. These surface receptors include cadherins and integrins. E-cadherins play the role of cell-cell adhesion and integrins play the role of cell-matrix adhesion. Genetic mutations cause cancer cells to lose surface receptors and hence lose the ability to adhere to neighbouring cells or matrix. This aids in the detachment of tumor cells from the primary tumor.

Once tumor cells detach from primary tumor, they migrate and intravasate into blood and lymphatic vessels [30] [31] (Figure 2-4). During intravasation, tumor cells undergo cellular deformation to transmigrate across the underlying basement membrane and endothelial cells of blood vessels [32]. Once the tumor cells intravasate into the circulation system of the body, they are termed circulating tumor cells (CTCs).

When the circulating tumor cells (CTCs) are in the blood, they are met with several obstacles. These include biological obstacles where the host immune system recognizes the CTCs as foreign pathogens and respond by releasing immune cells [33]. One particularly effective type of immune cell is the NK cell. NK cells have shown to induce murine immune response by providing effector molecules that are anti-tumoral [34]. Mechanical obstacle, in the form of hemodynamic fluid shear force by blood also plays an important role in destroying the CTCs. Hematogenous survival of the CTCs is very low because of the host immune response and the hemodynamic shear forces of the blood. Less than 0.01% of CTCs manage to survive and extravasate from the circulation [35].

Once these tumor cells extravasate into secondary sites of the body, they can exist in 3 forms. These 3 forms include solitary cells, micrometastases that lay dormant or metastases that immediately proliferate and vascularize. Only a portion of initial vascularized metastases are clinically detectable because of the relative small size in the initial stages and the imaging limitations from medical equipment; hence metastasis is the primary cause of high cancer mortality rate [5].

Figure 2-7 Summary of metastatic process. (A) Degrading of ECM and invasion. (B) Intravasation of tumor cells into circulatory system. (C) Circulating tumor cells (CTCs). (D) Platelet aggregation promotes adhesion to endothelial cells. (E) Extravasation. (F) Cancer cell settling at secondary site [101].

2.3 Tumor Heterogeneity

Tumor cells begin as a normal cell but after a series of alterations to its genes, the once normal cell proliferates faster, escapes the biological mechanisms which suppresses the enhanced growth or death and ultimately becomes invasive and metastasis. The everchanging and evolution of cancer cells do not stop even when they have become metastasis. The ongoing evolution of cancer cells creates heterogeneity because there are different cells at different stages of evolution. There are 2 kinds of heterogeneity and they are intertumoral heterogeneity and intratumoral heterogeneity.

Intertumoral heterogeneity is the difference of cells between different patients. This means that different patients with tumor growing in identical organs might have genetically different tumor cells. This makes treatment of cancer very difficult as different cells require different approaches.

Intratumoral heterogeneity refers to the difference among the tumor cells in a single patient. The genetic differences in tumor cells can be within a single site where the tumor resides or across different sites when the cancer have metastasized [36]. This heterogeneity makes studying cancer and their respective treatment very difficult too because most cancer therapeutic research uses different cell cancer lines without genetic variations that are already well used in the scientific community. This allows specific cancer cell line to be well studied but as mentioned, intratumoral heterogeneity exists in a single patient. Even before metastasis happens, various genetic differences stemming from the same origin cell line can be found at a single site.

During the course of tumor progression at the primary tumor site, non-malignant cells proliferate and gradually convert to malignant cells. The conversion is largely due to genetic alterations that allows cells to proliferate at a rate faster than normal cells, release angiogenesis factors to induce the growth of blood supply, escape apoptosis of cells and release factors that encourages metastasis [37] [38]. This conversion can be gradual and ongoing, and this generates a heterogenous tumor bulk as the tumor progresses and grows. Within this tumor bulk, there can exist cells from the same origin but of different genetic alterations and hence different molecular structures [36]. One of this molecular difference in breast cancer cell, in particular MCF-7 cells is the expression levels of E-cadherins on the surface of the cells. As mentioned in the previous section, the reduced expression in E-cadherin in MCF-7 has shown a reduction of cell-cell adhesion and an increase in metastasis.

2.4 E-Cadherins

Cadherins are calcium-dependent adherent proteins found on cellular membrane (Figure 2-5). These molecules are involved in the cell-cell adhesion by forming homotypic interactions between cells. These interactions are stabilized by calcium ions. Calcium ions bind to cadherin to cause a conformation change to ensure structural stability and hence able to form cell-cell adhesions [39]. The concentration of calcium also affects the expression levels of E-cadherin present on surface of epithelial cells [40]. There are many different types of cadherins which differ in structure and functions. The E-cadherin is one of the most studied members of the type 1 cadherin family.

Figure 2-8 Epithelial cells adhering to each other through the homotypic interaction between E-cadherin chains (blue molecules) with the aid of calcium (Ca^{2+}) ions .

This particular cadherin (E-cadherin) is involved in the cell-cell adhesions [41] [42] between epithelial cells and this is important as carcinoma (type of cancer) is of epithelial cells origin. In general, lower expression levels or suppression of cadherin activity in cancer cells allow these cells to be more invasive and infiltrates into other tissues because of the reduced cell-cell

adhesion [43] [44] [45]. Likewise, for breast cancer cells (carcinoma), the expression levels of E-cadherins have an inverse relationship to their invasiveness [46] [47]. The lower the expression levels of E-cadherin in carcinoma, the least adhesive between the cells. The reduced adhesion between the cells promote cancer cell dissemination which is the metastasis of cancer [48].

2.5 Integrins

Integrins are another group of surface receptor on cells. Unlike cadherins which aid in the cell-cell adhesion, integrins are in charge of attaching cells to the extracellular matrix components such as fibronectin. Similar to cadherins, the binding of the integrins to extracellular matrix components such as fibronectin requires the presence of ions, Calcium (Ca^{2+}) or Magnesium (Mg^{2+}) ions (Figure 2-6). The presence of either of these 2 divalent cations would activate and cause a stable heterotypic binding of the cell to extracellular matrix [49]. In the culture medium of MCF-7, which consists of DMEM, FBS and Penicillin, there are both calcium (Ca^{2+}) and

Figure 2-11 Extracellular Domains of Integrins with active site for divalent ions to bind to form stable bonds with extracellular matrix components

magnesium (Mg^{2+}) ions. These 2 divalent ions allow the cells to be involved in both cell-cell and cell-ECM adhesions. For cell-cell adhesions in MCF-7 cells, calcium ions are crucial. Any alteration to the calcium ions concentration would cause a change in expression levels of E-cadherins hence affecting the cell-cell adhesions. However, the alteration of calcium ions concentration do not affect the integrins because the integrins can depend on magnesium ions to form a stable heterotypic bind to components of the Extracellular matrix such as fibronectin. Besides the adhesion, integrins are also responsible for the cells' response to extracellular matrix which aids in tumor cell's invasion and migration [50].

2.6 Effects of Calcium concentration on cells

Calcium ions play a critical role in signal transduction, growth and differentiation in cells [51] [52]. This signal transduction is regulated both by intracellular and extracellular calcium ions. There are about 10,000-20,000 folds lower intracellular calcium concentration relative to extracellular calcium concentration [52] [53]. This difference in calcium concentration forms a gradient across the plasma membrane and the flow of calcium ions across plasma membrane is through a transmembrane calcium channel. The net flow of calcium would induce signaling pathway in the cytoplasm of the cells that triggers other intracellular activities.

Besides signal transduction, calcium concentration also affects cell growth and differentiation. It has been shown in studies that MCF-7 cells' shapes changes when the intracellular calcium concentration is significantly lower than normal. In the study presented by B Vandewalle and group, MCF-7 cells were grown in low extracellular calcium concentration environment (0.4mM) and high extracellular calcium concentration environment (2.5mM). MCF-7 cells grown in the lower extracellular calcium concentration environment presented cells' shapes that were rounded and 3 times smaller than cells cultured in higher calcium concentration [54]. Another study by Jonathan Pottle and group studied the effect of reducing intracellular calcium concentration on proliferation and growth of MCF-7 cells. The results showed that when there is a reduction or blockage in transmembrane calcium channels, there is a reduction in intracellular calcium concentration. This causes an increase in MCF-7 cells in G1 phase but a decrease in MCF-7 cells in S phase [55]. MCF-7 cells are dependent on calcium for proliferation and growth. This means that MCF-7 cells cultured in high calcium concentration environment will proliferate faster with more cells in a given time.

2.7 Conventional Methods to study Cancer

There were multiple conventional *in vitro* methods to study cancer cells. Some of these conventional methods include the Boyden chamber, micropipette assay and the wound healing assay (Figure 2-7).

The Boyden chamber (Figure 2-7 (A)), also called the transwell systems is traditionally used to characterize the invasive and migratory properties of single cell. In this assay, cancer cells are placed in a transwell insert which has porous membrane. ECM proteins such as fibronectin or collagen type I can be placed on top of the porous membrane to simulate ECM matrix. Cancer cells are then seeded onto the transwell insert before placing this insert into a well filled with chemoattractant. Chemical gradient is established between the transwell insert and the well. Cancer cells will transmigrate under the chemical gradient. The number of migrated cells is used as a metric to measure the invasive and migratory properties of the cancer cells [56]. The Boyden chamber assay is an easy and convenient way to investigate the invasiveness of cancer cells. This assay allows for live cell imaging. However, there are multiple limitations to this assay. The Boyden chamber cannot fully encapsulate the effect of a 3D microenvironment around a tumor. One example would be the coating of the transwell insert with ECM proteins. Although this process does increase the similarity of a tumor microenvironment by mimicking ECM proteins, it still does not fully translate the 3D microenvironment around tumor. Studies have shown that multiple features in a 3D microenvironment of tumor can simultaneously alter the invasive and migratory properties of cancer cells [57]. These features such as the pore size, stiffness and fibre orientation of ECM, the interstitial fluid pressure and interstitial flow velocity driven shear stress are unable to be replicated on a 2D substrate, and hence Boyden chamber assay has limitations in investigating and understanding cell invasion and migration.

The micropipette assay (Figure 2-7 (B)) is another 2D conventional method to investigate the migratory response of cell to chemokine gradients by using a micromanipulator-controlled micropipette to dispense predefined chemokine solutions. These chemokine solutions are released near the cells that are seeded on a 2D substrate. The limitation of this assay is that the chemokine gradient generated by micropipette is transient and difficult to quantify hence this assay is not the most ideal for investigating and understanding cell migration properties [57].

The wound healing assay (Figure 2-7 (C)) is used to study collective cell migration. An area without cells is generated in a cellular monolayer with the use of a cell scraper [58] or a micropipette [59]. The cells would collectively migrate and cover up the area without cells. The time is monitored to quantify the migratory properties of the cells.

Figure 2-13 Conventional in vitro methods to investigate cell migration. (A) Boyden chamber or the transwell systems is traditionally used to characterize the invasive and migratory properties of single cell. (B) The micropipette assay is a 2D conventional method to investigate the migratory response of cell to chemokine gradients by using a micromanipulator-controlled micropipette to dispense predefined chemokine solutions. (C) The wound healing assay is used to study collective cell migration [57].

2.8 Microfluidics used to study Cancer

With the emergence of microfluidic devices, biological research can now be conducted at a microscale. These microfluidic devices can resolve many limitations that are associated with conventional methods. Microfluidic devices can be customized according to experimental needs. Compartmentalization can also be achieved in microfluidic devices and this allows for spatial control and interaction between different cell distributions in a single experimental setup. Microchannels in microfluidic devices permit fluid flow which allows for the study of biomechanical processes such as shear stress [60]. Microfluidic devices are commonly made using the soft-lithography method and employ the use of a polymer called polydimethylsiloxane (PDMS). PDMS is applicable for biological studies because of its optical transparency which offers researchers opportunity to capture real time imaging, gas permeability which allows essential gaseous exchange to take place in the presence of biological substances and its biocompatibility with numerous biological substances [61]. Microfluidic devices using PDMS has multiple benefits in the biological and biomedical applications because they are cost-effective, has low consumption of biochemical reagents [62] due to its micro-scale dimensions and high spatiotemporal resolution that allows 3D observations of biological substances [63]. Microfluidic devices have been utilized extensively by many to study the metastatic cascade of cancer because of the above-mentioned advantages over conventional 2D methods.

Comparing with *in vivo* models, these microfluidic devices have optical advantages, better reproducibility and the ability of controlling the local environment around cells.

Among the metastatic cascade of cancer, the migration process of cancer under interstitial flow is very applicable to be studied with the use of microfluidic devices. This is because microfluidic

devices allow for the precise application of flow and pressure at a micro-scale, hence creating mechanical forces and generating chemical gradient on the cancer cells, alike *in vivo* environment. It also allows for the application of 3D microenvironment for the cancer cells which replicates tumor *in vivo*.

In 2012, Melody Swartz and her team of researchers designed a microfluidic device to study the migration behavior and response of a highly metastatic breast cancer cell line, MDA-MB-231 under constant interstitial flow (Figure 2-8) [64]. This microfluidic device contains 2 regions for different ECM materials that will be held by surface tension from the PDMS pillars. This allows different hydraulic permeabilities to be incorporated together by varying the concentration of the ECM materials. Different hydraulic permeabilities can be useful in setting different flow rates without changing the design of the microfluidic device. On each side of the compartments that hold the ECM materials are regions to contain medium. This device is designed such that flow is pressure driven by differing heights of 2 medium reservoirs (Figure 2-8 (D)). The pressure difference is maintained by a peristaltic pump. Melody Swartz and her team of researchers concluded from this study that the number of migrated cells and the migration speed of 20% of cells increase under interstitial flow of $10\mu\text{m/s}$.

Figure 2-14 Melody Swartz and team's design of microfluidic device to study the migration dynamics of breast cancer cell [64].

In 2009, Sudo *et al.* designed a microfluidic device to study the angiogenesis process of rat and human microvascular endothelial cells (rMVECs/hMVECs) in co-culture with rat's hepatocytes (Figure 2-9) [65]. In this design, a region consisting of a gel scaffold is held together by PDMS pillars through capillary forces. Two parallel microfluidic channels run top and bottom of the gel region. Cells can be introduced into each microfluidic channel and cultured on the gel region. In their study, the cells are cultured either under static or flow conditions (Figure 2-9 (B)). Under static conditions, droplets of culture medium are placed at either side of each channel. Under flow conditions, reservoirs are placed on either side of the microfluidic channel. Interstitial flow across the hydrogel region is generated by pressure difference between the two microfluidic channels. This pressure difference is maintained through reservoirs with differing heights of culture medium (Figure 2-9 (C)). Direction of interstitial flow can be manipulated by changing the positions of the reservoirs.

Figure 2-17 Sudo and team's design of microfluidic device. (A) Schematic diagram and dimension of device. (B) Cells culturing under static or flow conditions. (C) Pressure driven flow across gel region [65].

This study found that during the cell culturing and seeding period, an initial period of forward flow (Culture medium flow from microchannel with cells to microchannel without cells) followed by reverse flow (Culture medium flow from microchannel without cells to microchannel with cells) enhances the attachment of cells to the gel region and aided the formation of 3D tissue-like structures (Figure 2-10 (A)). In comparison, cells spread on the bottom surface of the microfluidic channel instead of being seeded on the gel region when reverse flow condition is applied from the beginning (Figure 2-10 (B)). This shows that interstitial flow plays a key role in the formation of 3D tissue-like-structures *in vitro*.

Figure 2-18 Effect of direction of flow on cell culturing and cell seeding. (A) Phase-contrast images of hepatocytes initially cultured under forward flow followed by reverse flow. Hepatocytes organized into 3D tissue-like structure and attached to the gel. (B) Phase contrast images of hepatocytes cultured in reverse flow from the beginning. Hepatocytes do not form 3D tissue like structures but instead form 2D thin sheets [65].

In this study, Sudo and his team seeded two different cells on either side of the gel region. They are the murine primary hepatocytes and rat/human microvascular endothelial cells (rMVECs/hMVECs). The team wanted to analyze the angiogenesis process in 3D cultures. The results show that when murine primary hepatocytes are co-cultured with rat microvascular endothelial cells (rMVECs), the murine primary hepatocytes undergo differentiation and the rat microvascular endothelial cells (rMVECs) penetrated the gel and formed vascular sprouts on the other side of the gel extending towards the murine primary hepatocytes (Figure 2-11). Prior to this co-culture setup, the rMVECs was cultured in the absence of murine primary hepatocytes and the mono-culture of rMVECs did not display any sprouts but instead form 2D structures. The team suggests that there is an interaction between the two cells through the diffusion of soluble proteins and signal molecules across the gel, promoting capillary morphogenesis of rMVECs in co-culture. The design of the microfluidic device is simple yet effective because it allows for the co-culture of two different cell types and interaction through a porous gel region. The permeability of the gel can be tuned by manipulating the concentration of the material. This study is encouraging because it shows the possibilities of co-culturing two different cell types in a microfluidic device with interaction and that interstitial flow plays a key role in creating 3D tissue-like structures *in vitro*.

Figure 2-22 Hepatocytes rMVECs co-culture. Phase contrast showing vascular sprouts [65].

In 2012, Shin and team improved on Sudo design by introducing a third microfluidic channel and gel injectors (Figure 2-12) [66]. The improved device allows for more than 2 cell types to be co-cultured together and the gel injectors allow for easier introduction of gel material into the design. This device encourages the study of interaction between multiple cell types. This is a robust device.

Figure 2-24 Shin and team's design of microfluidic device with 3 microchannels and gel injectors. (a) Schematic drawing of the microfluidic device. (b) Photograph of the microfluidic device [66].

In 2013, Kalchman and team designed a microfluidic device to study the effect of constant interstitial flow and anti-migratory drugs (Figure 2-13) [67]. The migratory response of human liver tumor cells (HCC) was studied under pressure driven flow across the collagen type I gel. The team suggested that HCC predominantly migrate against the flow direction and has an increase in proliferation rate in the presence of interstitial flow.

Figure 2-25 Kalchman and team's design of microfluidic device. (a) Schematic diagram of microfluidic device. (b) Photograph picture of microfluidic device with reservoirs attached to the ports of the microchannels to create pressure gradient across the gel region . [67].

2.9 Dielectrophoresis

Dielectrophoresis (DEP) is an effect where suspended particles move in a mixture due to the non-uniform electric field creating a polarizing force [68]. DEP causes particles to move through the polarization effects under a non-uniform electric field. DEP could be classified as positive or negative. In positive DEP, the particles have higher permittivity than the suspending medium and the particle move towards region of stronger electric field. On the other hand, the particle moves away from the region of stronger electric field for negative DEP [69].

Hence, with the use of DEP, it is possible to focus and immobilize cells in a microfluidic chamber for detection. Furthermore, live and dead cells have significantly different electrical properties which would then enable the identification of viable and non-viable cells in water sample [70] [71] [72].

Over the years, dielectrophoresis has evolved. In recent years, many has coupled dielectrophoresis (DEP) with microfluidics labs-on-chip, and this coupling has developed and opened up a multitude of novel applications in biomedical field.

The application of DEP in the biomedical field is very popular because DEP allows for biological substances to be further post processed because DEP does not permanently alter or change the characteristics both physically and biologically unlike other methods. Some of the applications of DEP on biomedical fields include but not limited to are cell sorting [73] [74], separation of live and dead bacteria or yeast [70] [71] [75], bacteria and virus detection [76] [77].

Bacteria forming pearl chains were observed for the first time in late 2019 to increase their surface area, and to achieve optimal food intake and storage from the environment to be

ecologically successful [78]. The group of researchers from the Max Planck Institute for Marine Microbiology in Bremen studied the *flavobacterium*, a Gram-negative bacterium that is ubiquitous in the North Sea thriving under nutrient-poor oligotrophic environment. Furthermore, another Gram-negative bacterium –*Thiomargarita namibiensis*, or more aptly named “Pearl of Namibia” is the largest bacterium ever discovered with diameter up to 750 μm [79]. The *Thiomargarita namibiensis* bacterium form chain of cells with light refracting sulfur globules that shines like a string of white pearls [79]. Interestingly, when bacteria, cells or particles are subjected to dielectrophoresis, they too have the tendency of forming pearl chain spanning across the microelectrodes gap of opposite polarities.

The ability to form human cancer cell pearl chain, down to the single cell level, would enable a robust platform for fundamental studies and clinical applications such as investigation of drug uptake or resistance, on-chip single cell labeling, and cell to cell adhesion strength. Another intriguing application of cell DEP for single cell isolation of cancer cells through negative DEP was to measure and characterize the cell impedance for identification of the pathological stages of cancer of MDA-MB-231 and MCF-7 [80]. Another application of DEP on cell is the separation of cancer cells from blood [81] and separation of different cancer cell types [82].

2.10 Research Scope

From the above-mentioned literatures, constant pressure driven flows are introduced using syringe-like devices across the microchannels. This was done by using reservoirs of differing heights to create pressure difference, hence generating interstitial flow across the microchannels. Constant pressure difference between the microchannels must be maintained for constant flow. Common practice to maintain constant pressure difference is to refill the reservoirs. This method is transient due to the evaporation of the medium in the reservoirs. This leads to differing flow velocity across the microchannels.

The above-mentioned literatures focus on the migration and behavior of cancer cells under the effect of constant pressure driven flow. Previous studies have found that interstitial flow creates gradients of biological signals and growth factors hence having effects on the migration and behaviors of tumor cells. Interstitial pressure difference that cause the fracture and masses of cells to dislodge, kickstarting the metastatic cascade has been studied on MCF-7, a breast cancer cell line. However, using different intercellular adhesivity of MCF-7 replicating *in-vivo* tumor heterogeneity [83] to study the effects of interstitial pressure difference that cause fracture in masses of cells on is still not well studied. Besides, the effects of increasing extracellular matrix component which in turn increases the cell-extracellular matrix strength in the tumor microenvironment is also not well studied.

In this study, the intercellular adhesivity of MCF-7 is varied by altering the culturing medium. The varying intercellular adhesivity will be replicated by culturing MCF-7 cells in different calcium ions concentration culture medium. This will affect the stability of E-cadherins which will affect intercellular adhesions of MCF-7 cells when forming spherical aggregates. Spheroidal

cellular aggregates will be prepared externally and introduced into the microfluidic device. Spheroidal cellular aggregate in the microfluidic device mimics a primary tumor. Interstitial flow with increasing flow rate will be introduced to the cellular aggregate using syringe pumps connected to the microfluidic device via rubber tubings. The pressure difference that causes fractures and eventually masses of cells to dislodge using varied intercellular adhesivity MCF-7 under interstitial fluid pressure is recorded by digital manometer. I propose to study the detachment of MCF-7 with varying intercellular adhesivity under interstitial pressure and flow to study the heterogeneity in tumor.

I will also be altering the fibronectin concentration for the coating on the microfluidic device to mimic the varying extracellular matrix component to study the effects of interstitial flow on the increase cell-extracellular matrix strength. Same microfluidic setup and pressure readings as above will be taken to measure the pressure difference that cause fracture.

Then, I would employ the use of dielectrophoresis on MCF-7 cancer cells. This will allow me to visualize the formation of human breast cancer cell (MCF-7) chain under positive and negative dielectrophoretic force.

In the following section, the methods and materials required for this study will be explained thoroughly.

3 Chapter 3 – Microfluidics modeling of interstitial flow through a tumor

3.1 Methods and Materials

3.1.1 Microfluidic Device

The benefits of microfluidic devices have been studied and documented extensively [61]. The author used soft lithography method to fabricate the microfluidic devices used in this research.

First, photoresist SU8-2100 was spin-coated onto a silicon wafer. The SU8-2100 photoresist is a negative photoresist. Negative photoresists are materials that become insoluble in developing solutions after being exposed to ultra-violet radiation (Figure 3-1) [84]. Next, photo-lithography method was used to transfer the desired design onto the photoresist. Then, the coated silicon wafer with the desired design was treated with a developer which is a chemical that removes the unexposed photoresist. The microchannel designs were left behind after the developing process. A thickness of 100-110 μm was used for the thickness of the microchannels.

Figure 3-1 Steps in Photo-lithography.

Polydimethylsiloxane (PDMS) was mixed in the ratio of 10:1 between PDMS monomer and curing agent. The PDMS was poured onto the wafer with the desired design created by developing the negative photoresist SU8-2100. A 5 mm thick PDMS chip is formed. After curing, holes were punched through the PDMS at the inlet sites to each microchannel. The PDMS was subsequently bonded to a glass slide via plasma bonding. The assembled device was then soaked with distilled water and autoclaved at 110°C for 45 minutes (Figure 3-2).

Figure 3-2 PDMS device after fabrication and plasma bonding..

The device consists of 5 microchannels with a square chamber of dimensions 500x500 μm in the center (Figure 3-3, Figure 3-4). This chamber holds the cellular aggregate for the experiment. Flow will be applied from channel 1 (input) to channel 5 (output). These channels are designed with a width of 300 μm . Before the implementation of the flow, the cellular aggregate is introduced into the square chamber via channel 3. The width of channel 3 is also designed to be 300 μm . At this width, the cellular aggregate is able to recover its shape easily after being introduced into the square chamber. 2 channels, channel 2 and 4, are connected perpendicular to the main channel with the flow. These 2 channels are connected to each other. The channel that connects these 2 channels is running parallel to the main channel. Channels 2 and 4 have a width of 50 μm wide and are used for measuring the pressure drop across the chamber while the flow is being applied. These two channels are connected at the inlet to two digital manometers to monitor the pressure difference across the chamber.

Figure 3-3 Dimensions of microchannels and square chamber (mm).

When a flow is applied to the input channel (Channel 1), a large portion of the input flow preferentially moves to the side channel due to a lower hydraulic resistance in the side parallel channel as compared to the main channel. The main channel has a higher hydraulic resistance because of the presence of the cellular aggregate. This results in a pressure difference at two ends of the side channel. This parallel-channel design means that the pressure difference across the side channel is equal to the pressure difference across the cell chamber. The hydraulic resistance of the side channel can be pre-determined.

Figure 3-5 Drawing of device designed in SolidWorks.

The hydraulic resistance is defined as:

$$\Delta P = Q \times R_h$$

where ΔP (Pa) is the change in pressure across the channel, Q (m³/s) is the flow rate along the channel and R_h is the hydraulic resistance.

The hydraulic resistance is inversely proportionate to flow rate $R_h \approx \frac{1}{Q}$, hence the pressure difference across the cellular aggregate can be calculated from the input flow rate and flow rate through the side channel.

3.1.2 Fabrication of DEP microfluidic chip

The microelectrodes were fabricated with standard cleanroom lithography techniques. First, the design of the microelectrodes was patterned onto a 4" Borofloat 33 glass wafer with positive photoresist (AZ9260, Microchemicals GmbH). Subsequently, a layer of 10 nm of chromium and 200 nm of gold was magnetron sputtered onto the glass wafer. The microelectrodes have a gap of 100 μm for breast cancer cell MCF-7 experiments. An open reservoir design was used for the breast cancer cell experiment for the ease of pipetting from cell culture. Furthermore, the open reservoir design adopted for the cancer cell experiment is to achieve no flow condition, where the movement of the cells are induced only by dielectrophoresis and gravitational force.

3.1.3 Measurement of Hydraulic Resistance across side channel

A series of experiment were conducted to measure the hydraulic resistance across the side channel. First, the main channel has to be blocked so that all the fluid flows through the side channel. To achieve this, PDMS solution (Mixed in the ration of 10:1 for PDMS Monomer and curing agent) was introduced into the square chamber through channel 3. The solution was then cured in an incubator for 2 hours. After 2 hours, the PDMS exists as a solid form in the square chamber obstructing flow in the main channel. All the fluid introduced into the device will now be flowing through the side channel.

A constant flow of $10\mu\text{l min}^{-1}$ is introduced into the device. Using equation (1) and the average pressure across the side channel of 299.78 Pa (Figure 3-5), the hydraulic resistance of the side channel is measured at $29.97\text{ Pa min } \mu\text{l}^{-1}$. This result is in line with previous study which use the same device.

Figure 3-6 Graph depicting pressure difference across side channel when introduced with constant flow rate of $10\mu\text{l min}^{-1}$

3.1.4 Verifying effectiveness of device in measuring porous media

There is a need to verify the effectiveness of the device in calculating the permeability of a material when placed in the square chamber using the pressure difference across the side channel.

This is done by introducing collagen type 1 coated with FITC into the square chamber (Figure 3-6). This collagen type 1 has a predetermined permeability. A constant flow rate of $10\mu\text{l min}^{-1}$ is applied, and pressure readings are recorded by manometers at the side channels (Figure 3-4). This whole procedure is similar to previous section except for the difference in material in the square chamber.

Figure 3-7 Collagen Type 1 mixed with FITC introduced into square chamber of device.

The pressure readings obtained from the side channels have an average of 267 Pa (Figure 3-7). Using Darcy's law of permeability across collage type 1 and the pressure change across the collagen type 1, the permeability of the collagen type 1 is found to be 5.47×10^{-12} [57] [85]. This is in the same order of magnitude as previous studies. From this, it is shown that the device is able to calculate the permeability well by measuring the pressure change across the side channel.

Figure 3-8 Pressure difference across side channel with collagen type 1 in a square chamber

Darcy's Law:

$$Q = \frac{kA}{\mu L} \Delta P$$

where Q is the volumetric flow rate (m^3/s), k is the permeability of the material (m^2), A is the cross-sectional area in the flow's path (m^2), μ is the dynamic viscosity ($\text{Pa} \cdot \text{s}$), L is the length of the material (m) and ΔP (Pa) is the change in pressure across the material.

3.1.5 MDA-MB-231

There are a variety of breast cancer cell lines that has varying degrees of aggressiveness. MCF-7, being the cell line used in this project is classified as not aggressive. This means that patients who are diagnosed with MCF-7 breast cancer has a better prognosis. Due to the heterogenous nature of cancer tumors *in vivo* [36], the best way to study the interstitial flow through a tumor aggregate is to use different cell lines as aggregates for the flow experiment. MDA-MB-231 being a well-studied and common breast cancer cell line that is more aggressive than MCF-7, it is chosen to be studied and used in this project [86].

The doubling time for MDA-MB-231 is ~ 38 hours [87]. In the first 2 weeks (4 passages), the cells proliferate at a rate slower than what was observed by others. This could be because these cells were cryo-preserved prior to culturing. After the cells were observed to be healthy and proliferating normally in culturing flask, different methods for aggregation were experimented on. Classification of MDA-MB-231 structures formed from aggregation was used (Figure 3-8) [88].

Figure 3-9 Classification of structures formed from aggregation [86].

In the first attempt, a concentration of 200,000 cells/ml was chosen to be orbital shaken in a 25-ml Erlenmeyer flask containing 5-ml of culture medium at 70rpm for 3 days to form MDA-MB-231 aggregate. This is the same method used in the aggregation of MCF-7. In the case of MCF-7, compact and large aggregates are formed. However, loose aggregates and singular cell suspensions of MDA-MB-231 were observed together (Figure 3-9). The average size of loose aggregate is approximately 80-100 μ m in diameter. This is 80% smaller than average MCF7 aggregate. The loose aggregates break up when pipette. This makes it hard to transfer into microfluidic device.

Figure 3-10 Loose aggregates (Blue) and singular cell suspensions (Red) of MDA-MB-231.

In the second attempt, a concentration of 50,000 cells/ml was chosen to be orbital shaken at 60rpm in a Cellstar Cell-repellent round bottom well plate for 3 days to form MDA-MB-231 aggregate. 1 compact aggregate is formed per well (Figure 3-10). The cell aggregate appears to be compact after orbital shaking in a well plate. This could be because a well plate has a smaller volume (Figure 3-10 (A)). Aggregate formed has a larger diameter than the previous method at approximately 500 μ m in diameter. This is consistent to the diameter of the MCF-7 aggregates formed previously. This method of aggregation seems to be promising because the MDA-MB-231 aggregates formed are compact and have good sizes to be used in the flow experiment. However, the aggregate's shape change and some parts of the aggregate broke apart after pipetting (Figure 3-10 (B)).

Figure 3-12 Compact aggregate form by orbital shaking in a well plate. (A) Compact aggregate. (B) Shape changed with some loss in cells from the original cellular aggregate after pipetting.

In the third attempt, 3.5% matrigel was added into both 25-ml Erlenmeyer flask containing 5-ml of culture medium with 400,000 cells/ml and into Cellstar Cell-repellent round bottom well plate with 100,000 cells/ml prior to orbital shaking at 60rpm for 3 days. Addition of 3.5% Matrigel method to obtain compact aggregate was a method employed previously by Schmidt's team [88]. Compact aggregate was formed in both the flask (Figure 3-11 (A)) and well plate (Figure 3-11 (B)). The size of aggregate is approximately 500 μ m in diameter. The compact aggregates formed with orbital shaking in the Erlenmeyer flask presented a coat around the aggregate. This coat was also observed in the cellular aggregate of MCF-7. This was not observed in other cellular aggregates done above. This could be due to the higher cell density used. Shape also changes upon pipetting, but no part of the cell broke apart. Since no part of MDA-MB-231 broke apart and any cells lost after pipetting, MDA-MB-231 can potentially be used in the flow experiment.

Figure 3-14 Compact aggregate is formed. (A) Compact aggregate formed from orbital shaking in well plate with 12 μ l of matrigel and 100,000cells/ml. (B) Compact aggregate formed from orbital shaking in flask with 10 μ l of matrigel and 400,000cells/ml.

Using this method of aggregation, MDA-MB-231 aggregate was introduced into the microfluidic device to test the viability of these cellular aggregate in the experiment.

While introducing the aggregate into the chamber of the microfluidic device by introducing a small flow using syringe pump connected from to channel 1 at a rate between (20-50 μ l/min), the cellular aggregate immediately broke up (Figure 3-12). This could be because MDA-MB-231 has low E-cadherin expression levels. With low levels of E-cadherin, the cellular aggregate has weak intermolecular bonds. MDA-MB-231 aggregates are compact but do not form spheroids and have weak intermolecular bonds. Hence, using MDA-MB-231 as cellular aggregate for flow testing is not feasible. With this, another approach to mimic tumor heterogeneity is being considered.

Figure 3-15 MDA-MB-231 in square chamber. The cellular aggregate broke apart when introduced with a flow from the inlet into the square chamber. This is due to the low expression of E-cadherin.

3.1.6 MCF-7

To investigate the effects of e-cadherin expression, there is a need to use different cell lines with different extent of cancer aggression as materials for the experiment. As shown in the previous section, using MDA-MB-231 is not desirable as the cells do not adhere to each other strong enough to be introduced into the square chamber for the experiment. This is understandable as less adhesive cell lines are associated with more aggressive cancers.

MCF-7 (Michigan Cancer Foundation 7) breast cancer cell line was used in this study. The cells were cultured in a 75cm³ culture flask and incubated in a 5% CO₂ environment. The culture medium used in this study to culture the cancer cells, consisted of 89% Dulbecco's Modified Eagle Medium (DMEM), 10% Fetal Bovine Serum (FBS), and 1% Penicillin-Streptomycin.

To vary the adhesion levels of MCF-7 to mimic more aggressive cancer cell lines, the stability of E-cadherins can be altered. This can be done by varying the concentration of calcium ions in the culturing medium and ultimately affecting the expression of E-cadherins on MCF-7 [39] [89] [90] [54] [40].

There is a need to first explore the calcium concentration in each of the constituents of the culture medium, namely Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS) and Penicillin-Streptomycin.

Dulbecco's Modified Eagle Medium (DMEM) that is used in this research is purchased from ThermoFisher Scientific. According to the company's formulation of DMEM, calcium is found as calcium chloride anhydride(CaCl₂), an inorganic salt. The concentration of calcium ions is listed as 1.8mM, which is equivalent to $1.8 \times 10^{-3} \text{mol/L}$ of DMEM [91].

Fetal Bovine Serum (FBS) used in this research is purchased from ThermoFisher Scientific The average concentration of calcium ions in FBS is 6.75µeq/ml which is equivalent to $3.375 \times 10^{-3} \text{ mol/L}$ of FBS [92].

Penicillin-Streptomycin used in this research has no calcium ions present.

From the listed concentrations of calcium ions (mol/L) in each chemical and the specific amount of chemicals used to make 100ml of culture medium for MCF-7, the concentration of calcium ions in each constituent of culture medium can be calculated with the following equation.

$$\begin{aligned} \text{Conc of } Ca^{2+} \text{ in chemical } \left(\frac{\text{mol}}{L} \right) \times \text{Amount of chemical used (L)} \\ = \text{Conc of } Ca^{2+} \text{ in culture medium} \end{aligned}$$

DMEM:

$$1.8 \times 10^{-3} \left(\frac{\text{mol}}{L} \right) \times 0.089 (L) = 0.1602 \times 10^{-3} \text{ mol}$$

FBS:

$$3.375 \times 10^{-3} \left(\frac{\text{mol}}{L} \right) \times 0.010 (L) = 0.0334 \times 10^{-3} \text{ mol}$$

Summing up, the amount of calcium ions in 100ml of culture medium is $0.1936 \times 10^{-3} \text{mol}$, equivalent to 1.936mM. Armed with this knowledge, varying the concentration of calcium ions to affect the stability of E-cadherins on MCF-7 can be achieved with the addition of calcium absent DMEM (DMEM-). The following table shows the corresponding amount of mixture between DMEM- and amount of culture medium (89% DMEM, 10% FBS and 1% Penicillin-Streptomycin) to form culture medium for both cell culturing (12ml) (Table 1) and cell aggregation (5ml) (Table 2) with various levels of calcium concentration.

Table 1: Required volume of culture medium and DMEM- to make different calcium concentration culture medium for cell culturing (12ml)

Ca²⁺ Concentration (mM)	1.0	1.2	1.4	1.5	1.6	1.7	1.8	1.9
Culture Medium Volume (ml)	6.199	7.438	8.678	9.072	9.917	10.536	11.158	12.00
DMEM- Volume (ml)	5.801	4.562	3.322	2.928	2.083	1.464	0.842	0

Table 2: Required volume of culture medium and DMEM- to make different calcium concentration culture medium for cell aggregation (5ml)

Ca²⁺ Concentration (mM)	1.0	1.2	1.4	1.5	1.6	1.7	1.8	1.9
Culture Medium Volume (ml)	2.583	3.099	3.616	3.780	4.132	4.390	4.649	5.000
DMEM- Volume (ml)	2.417	1.901	1.384	1.220	0.868	0.610	0.351	0

Cell aggregation in this study was done with some modifications to Ryan *et al.* method [93]. The cell aggregates were produced when the MCF-7 in the culture flask reaches confluency. To produce the cellular aggregate, the author first treated the confluent cells in the culture flasks with 0.25% Trypsin/0.53 mM EDTA solution. After 3-5 minutes of treatment, culture medium was employed to deactivate the trypsin by mixing it in the ratio of 3:5 between Trypsin and Culture medium. The cells were then centrifuged at 1500rpm for 7 minutes. After centrifugation, supernatants were discarded and MCF-7 pellet is re-suspended with culture medium. Adequate number of cells are transferred to a 25-ml Erlenmeyer flask containing 5 ml of CO²-equilibrated culture medium at a concentration of 2×10^5 cell ml⁻¹. The Erlenmeyer flask was then placed inside an incubated orbital shaker at 70 rpm for 24 hours. The temperature in the shaker was set at 37°C to mimic body temperature. After 24 hours, the breast cancer cells aggregated and formed spheroidal shapes with diameters ranging from 50 to 450µm (Figure 3-13 (A)). When conducting the experiment, these cell aggregates were chosen by selection of size through a microscope. Selected cellular aggregates have sizes about 450µm (Figure 3-13 (B)).

Figure 3-16 Cellular aggregate of MCF-7. (A) Cellular aggregate generated from with orbital shaking. The sizes of the cellular aggregates are non-uniform. (B) Chosen cellular aggregate for the experiment. The chosen aggregate's diameter is ~500µm.

After culturing MCF-7 in 1.5mM and 1.9mM calcium concentration culture medium, cell size of MCF-7 cultured in the 2 different medium is measured using ImageJ to compare the cell morphology.

Using ImageJ, microscopic images of MCF-7 cells (Figure 3-14 (A)) cultured on the surface of the flask is first converted to greyscale by changing the image type to 8-bit. After which, the background is subtracted to present a black and white image of cells. Next, thresholding is set at 12 pixels. 12 pixels is chosen to add contrast to the black and white image of cells and remove background noises, in the form of white specks (Figure 3-14 (B)). Third, watershed segmentation is done. This is an image processing step that introduces 1 white pixel in between 2 cells to allow for the cells to appear further apart and easier automation for cell count in the subsequent step. Last, the particle analysis function in ImageJ is employed. This step automatically counts the cells, cell area, aspect ratio and cell circularity. Outlines are set around cells that has already been counted so that checks can be done on the program.

Figure 3-20 MCF-7 cultured in 1.5mM calcium concentration culture medium on a culturing flask. (A) Image of MCF7 cells under brightfield microscope. (B) Identical image of MCF-7 cells in black and white after image processing using ImageJ.

3.1.7 Western Blotting

Cancer tumors are heterogenous. Within the tumor, there are various genetically different cells. These genetically different cells may differ in aggressiveness in terms of the ease at which single cells detach from the primary site and metastasize to distant sites. The easier it is for single cells to detach, the more aggressive the cancer is. One factor that determines the ease of single cells detachment from primary tumor is the cell-cell adhesivity which in turn is influenced by the expression levels of E-cadherin on the surfaces of the cells. The initial proposed method in this stage of the project was to manipulate protein expression of MCF-7 through the alteration of gene expression specific to the protein of interest - E-cadherin. This method allows for genetically identical cells to adhere differently to each other based on the levels of E-cadherin expressions on the cell surface.

Alteration of gene expression that is specific to the protein interest does not allow different levels of proteins to be expressed. It is an all-or-none situation. This is because alteration of gene expression to down-regulate proteins requires that particular gene to be removed through the process of splicing. This process completely removes the gene coding for E-Cadherin and hence as a result, completely eradicates the transmembrane protein E-cadherin protein from being produced.

In this project, western blot is employed to confirm the presence of E-cadherins in MCF-7 cells. This technique allows the detection of specific protein which is the E-cadherin, through the use of antibodies.

First, MCF-7 are lysed to release the protein of interest, E-cadherin. Cellular proteins are separated by the molecular weight via gel electrophoresis (Figure 3-15).

After separation, the proteins are transferred onto a nitrocellulose membrane. The nitrocellulose membrane with proteins is then incubated overnight in a mixture of primary antibodies that are specific to the protein E-cadherin and milk. The milk would bind to the other proteins of the cells which are of no interest in my project. The primary antibodies will bind specifically to E-cadherins. Subsequently, fluorescently tagged secondary antibodies which are specific to the primary antibodies' species are added and incubated. This allows for the protein of interest to be identified on a film after developing in a dark room (Figure 3-16).

Figure 3-21 Cellular proteins are separated by their molecular weight via gel-electrophoresis.

Figure 3-24 Film developed in dark room. The bands represent the presence of the protein of interest.

The intensities of the bands expressed on the film developed from western blot are first normalized with the intensity of a loading control Actin. During the primary antibody incubation period, the nitrocellulose membrane was cut into 2 and the portion with Actin protein was incubated with primary antibody specific to Actin (Figure 3-17).

Figure 3-27 Protein bands (above) and actin (below) from MCF-7.

The normalized results of the intensities of the bands can be used to semi-quantify the expression levels of E-cadherins in MCF-7. This is not as accurate as other testing methods as the results depend heavily on the consistencies in the various steps done in the above-mentioned procedure. Hence, western blotting was not pursued further to quantify and compare the expression levels of E-cadherins in the cell lines that are used in this study.

3.1.8 Enzyme Linked Immunosorbent Assay (ELISA)

The enzyme linked immunosorbent assay (ELISA) is a test that enables the quantification of protein using antibodies. The Human Total E-Cadherin ELISA Kit (DYC4225-2) purchased from RnD Systems is employed in this study to specifically quantify the expression levels of E-cadherin in MCF-7 that are cultured in different calcium concentration medium. The Human Total E-Cadherin ELISA Kit (DYC4225-2) is a sandwich ELISA kit.

First, 100µl of diluted immobilized capture antibody that is specific to the human E-cadherin protein is coated on the bottom surface of a 96 well-plate and left incubated at room temperature overnight. Next day, the wells are washed 3 times, blocking buffer is added and left for 2 hours. Concurrently, monolayer MCF-7 cells cultured in 1.5mM and 1.9mM calcium ion concentration culture flask are trypsinized and counted for cell density. The cell densities would determine the amount of lysis buffer added. For every cell passage, the average cell densities present in each culture flask is about 13million cells. The cell densities differ from passages to passages and from flask to flask. The cells are centrifuged and washed with PBS 3 times. After which, the required lysis buffer is added here according to the manufacturer of the ELISA kit and mixed well by vortexing for 20secs and iced for 15mins. 500µl of lysis buffer is needed for every 5 million cells. Then, the blocking buffer is aspirated and washed 3 times again. 100µl of lysed samples that were prepared based on manufacture's recipe and 100µl of diluted standards are added to the wells. The standards and samples are again left in the wells for 2 hours. After which, the standards and samples are aspirated and washed 3 times before diluted detection antibodies are added. The detection antibody is again left in the wells for 2 hours. Subsequently, the detection antibody is aspirated, wells washed 3 times and Streptavidin-HRP is added and left for 20 minutes in the dark. Repeat the aspiration step and wash 3 times before adding the

substrate solution and left to be incubated for 20 mins in the dark before adding the stop solution. The optical densities of the samples and the standards are measured at 450nm wavelength in a microplate reader and corrected at 540nm wavelength.

During the initial tries of conducting ELISA test, optimizations must be done. The number of cells across the samples must be identical to have an accurate comparison of E-cadherin levels across cells cultured in different culture medium. This number of cells must also be optimized so that the optical densities of the samples are within the range of the standards. A few runs were catered for these optimizations.

The optical densities of the samples are within range of the standards. For each standard protein concentration, 2 wells are utilised so that an average can be calculated. The average optical densities of zero protein concentration (Control) is deducted from each optical density of the protein concentration standards before average is calculated and recorded. Results table of average optical densities can be found and discussed in the results and discussion chapter.

3.1.9 Experimental Setup

Before starting the experiment, the microfluidic device was coated with bovine fibronectin solution. The bovine fibronectin solution with concentration of $100\ \mu\text{g ml}^{-1}$ was pipetted in the microchannels and left to incubate for 12 hours at 37°C . The microchannels are flushed with Phosphate-buffered Saline (PBS) solution after 12 hours of incubation. The microfluidic device is then ready for use in the experiment.

For the experimental setup, the coated device was placed on the microscope stage (Figure 3-18 (A)). Tubings were fitted into each inlet (Figure 3-18 (B)). These tubings were connected to different instruments for the experiment. At inlet 1, the tubing was connected to a syringe pump (Syringe Pump 1). This syringe pump dispensed a mixture of CO_2 -equilibrated culture medium and 1% of FITC fluorescein dye. At inlet 5, the tubing was connected to a reservoir. At inlet 3, the tubing was used to transport the cell aggregate into the square chamber via a syringe pump (Syringe Pump 2). At inlets 2 and 4, the tubings were each connected to digital manometers to measure the pressure difference across the cellular aggregate. The microchannels were filled with culture medium.

Figure 3-29 Experimental setup. (A) Microfluidic device setup with tubings and output reservoirs on microscope stage. (B) Microfluidic device with tubings inserted to various inputs of the microfluidic device's channel.

To start the experiment, the cellular aggregate was transported through channel 3 into the square chamber. A pipette was used to inject a selected cellular aggregate into the inlet of channel 3. Next, syringe pump 2 was connected to the inlet of channel 3. The microscope stage was kept at room temperature to prevent the adhesion of the cellular aggregate to the walls of microchannel before reaching the square chamber. A flow was applied to bring the cellular aggregate into channel 3. A clamp was used to control the flow rate entering inlet 3 to prevent the cellular aggregate from moving too fast into channel 3 and breaking up.

After the cell aggregate entered the square chamber, the microscope stage was adjusted to 37°C which mimicked the body temperature and static condition was maintained. The aggregate was left to sit for 2 hours without flow disturbance inside the chamber (Figure 3-19). This resting period was crucial for the cell aggregate to adhere to the fibronectin coated square chamber.

Figure 3-30 Cellular aggregate left to sit for 2 hours before applying flow.

After 2 hours, the cellular aggregate was observed to adhere to the channel walls by the change of color of cells adjacent to the wall of the PDMS. A flow of CO₂-equillibrated culture medium mixed with 1% of FITC fluorescein dye was applied to the cellular aggregate via channel 1. The digital manometers connected to channels 2 and 4 were then switched on at the same time to record the change in pressure difference across the cellular aggregate.

3.2 Results and Discussion

3.2.1 Morphological Difference between MCF-7 cultured in different calcium concentration DMEM

To understand if altering the calcium concentration ions in the culture medium for MCF-7 affects the morphology of MCF-7, the cell size, cell's aspect ratio and cell's circularity must be measured. These morphological parameters of MCF-7 cultured in 1.5mM and 1.9mM calcium concentration culture medium is measured using ImageJ as described in the method section above. The cells cultured in these 2 calcium concentration is chosen to study the morphological differences because they contains the least and most calcium concentration in culture medium in my experiment.

The doubling time for MCF-7 cultured in 1.9mM calcium concentration culture medium is 29 hours [94]. From day 7 onwards, cell growth reached plateau. This means that most cells do not undergo rapid cell cycling phases and hence most cells' sizes remain relatively constant from day 7 [95]. The cells cultured in both 1.5mM and 1.9mM calcium concentration culture medium which are used to measure the cell sizes, aspect ratio and circularity are collected on day 7 when the growth reached plateau.

In the 1.5mM calcium concentration culture medium, there are a total of 2280 cells in 0.06mm² surface area of the culturing flask after 168 hours. The smallest cell size is 0.32μm² and the largest cell size is 71.82μm². The mean cell size is 5.59μm² (Figure 4-1). In the 1.9mM calcium concentration culture medium, there are a total of 2485 cells in 0.06mm² surface area of the culturing flask after 168 hours. The smallest cell size is 0.32μm² and the largest cell size is 74.37μm². The mean cell size is 7.70μm² (Figure 3-20).

Comparing the mean sizes, MCF-7 cells cultured in 1.5mM calcium concentration culture medium is significantly smaller in size ($p < 0.05$) than MCF-7 cells cultured in 1.9mM calcium concentration culture medium with a mean difference of $2.138\mu\text{m}^2$ (Table 3). The mean size alone does not represent morphological changes because there are other parameters to be considered before morphological differences can be concluded.

Figure 3-31 Graph of size of MCF-7 cells cultured in 1.5mM vs 1.9mM calcium concentration medium

Table 3: T-test comparing cell size between MCF-7 cultured in 1.5mM vs 1.9mM calcium ions

T-Test

	t	df	p	Mean Difference	SE Difference	95% CI for Mean Difference	
						Lower	Upper
Area	-9.847	4762.178	< .001	-2.138	0.217	-2.564	-1.712

The smaller in size of MCF-7 cells cultured in 1.5mM calcium concentration could be because of different cell cycling periods. MCF-7 cells cultured in 1.5mM calcium concentration could have a longer cell cycling period. Both the MCF-7 cells cultured in 1.5mM and 1.9mM calcium concentration are harvested on day 7 which is previously documented as the day at which MCF-7 cells cultured in 1.9mM calcium concentration reach growth plateau and rapid cell cycling stops. The cell count numbers between MCF-7 cells cultured in 1.5mM and 1.9mM calcium concentration also agrees with this. There are ~10% fewer MCF-7 cells in the 1.5mM calcium concentration culture medium than in the 1.9mM calcium concentration culture medium. This shows that the cell cycling period is longer in 1.5mM calcium concentration.

The aspect ratio is another parameter to observe morphological differences in cells. In the 1.5mM calcium concentration culture medium, the mean aspect ratio is 2.20 with 21 data points being the outlier (Figure 3-21). These 21 cells represent <1% of the cells counted in the 1.5mM calcium concentration. These 21 cells are elongated as MCF-7 cells have the tendency to extend between clusters to cling onto each other (Figure 3-22(A)). In an area with fewer cells, there will be more cell extensions observed.

In the 1.9mM calcium concentration culture medium, the mean aspect ratio is 2.16 with 18 data points being the outlier (Figure 3-21). These 18 cells represent <1% of the cells counted in the 1.9mM calcium concentration. Similar to the cells in 1.5mM calcium concentration, these 18 cells are outliers that extends between clusters of cells to cling onto each other (Figure 3-22(B)). There are lesser percentage of elongated cells in the 1.9mM calcium concentration culture medium because the surface is more populated with cells as compared to 1.5mM calcium concentration. This means that there are bigger clusters and lesser gaps on the surface of the flask that contains the 1.9mM calcium culture medium.

Figure 3-34 Graph of Aspect Ratio of MCF-7 cells cultured in 1.5mM vs 1.9mM calcium concentration medium

Figure 3-38 Microscopic view of MCF-7 cultured on the surface of a flask. There are more elongated cells in 1.5mM calcium concentration culture medium as there are lesser cell clusters and hence, cells elongate between clusters to maintain contact. (A) MCF-7 cultured in 1.5mM calcium concentration culture medium showing elongated cells, represented by red triangle. (B) MCF-7 cultured in 1.9mM calcium concentration culture medium showing lesser elongated cells.

From the t-test results (Table 4), the aspect ratio between MCF-7 cultured in 1.5mM and 1.9mM calcium concentration culture medium does not show significant differences ($p > 0.05$). Despite having more elongated cells in the 1.5mM calcium concentration, the rest of the MCF-7 cells in the 1.5mM calcium concentration makes up for it and hence there are no significant difference of aspect ratio between the cells cultured in 1.5mM and 1.9mM calcium concentration culture medium.

Table 4: T-test comparing cell aspect ratio between MCF-7 cultured in 1.5mM vs 1.9mM calcium ions

T-Test						
	t	df	p	Mean Difference	SE Difference	95% CI for Mean Difference
						Lower Upper
Aspect ratio	1.236	4608.163	0.217	0.039	0.032	-0.023 0.102

To further check the shape of MCF-7, the circularity of the cells is measured. For circularity, a value of 0 represents completely not circular and a value of 1 represents a complete circle. The mean value of circularity for MCF-7 cells cultured in 1.5mM calcium concentration culture medium is 0.63 and the mean value of circularity for MCF-7 cells cultured in 1.9mM calcium concentration culture medium is 0.57 (Figure 3-23). The mean values of circularity of MCF-7 cells cultured in both 1.5mM and 1.9mM calcium concentration culture medium agrees with the aspect ratio that the shape of the MCF-7 cells cultured on the surface of culturing flask possess the shape of an ellipsoid.

Figure 3-39 Graph of circularity of MCF-7 cells cultured in 1.5mM vs 1.9mM calcium concentration medium

Table 5: T-test comparing cell circularity between MCF-7 cultured in 1.5mM vs 1.9mM calcium ions

T-Test							
	t	df	p	Mean Difference	SE Difference	95% CI for Mean Difference	
						Lower	Upper
Circularity	6.461	4663.489	< .001	0.061	0.009	0.042	0.079

However, from the t-test results (Table 5), the cell circularity between MCF-7 cultured in 1.5mM and 1.9mM calcium concentration culture medium shows significant difference ($p < 0.05$). Although MCF-7 cells cultured in 1.5mM calcium concentration culture medium is ellipsoid alike MCF-7 cells cultured in 1.9mM calcium ions concentration culture medium, they are generally more circular in shape. This result agrees with the aspect ratio because despite having more elongated MCF-7 cells in the 1.5mM calcium concentration culture medium, the more circular shape of the rest of the cells gives the mean aspect ratio a value significantly similar to the MCF-7 cells cultured in 1.9mM calcium concentration culture medium.

The results of cell size, cell aspect ratio and cell circularity between MCF-7 cells cultured in both 1.5mM and 1.9mM calcium concentration culture medium shows that MCF-7 cells cultured in 1.5mM has a significant difference in mean cell size and cell circularity but the aspect ratio does not have significant difference.

Considering the cell size, cell aspect ratio and cell circularity between MCF-7 cells cultured in 1.5mM and 1.9mM calcium concentration culture medium, it can be concluded that there are morphological changes to MCF-7 when cultured in difference calcium concentration. This is expected as a change in calcium ion concentration in the culture medium for MCF-7 affects the culture environment. With a change in culture medium, the morphology of MCF-7 will be affected.

The project's focus is on the effects of interstitial fluid pressure on different intercellular adhesivity of MCF-7 to study the heterogeneity that exists in tumor. The difference in intercellular adhesivity is done by altering the calcium concentration in culture medium which would affect the stability of E-cadherins. This change in calcium concentration to the culture medium also cause morphology changes to MCF-7 cells. This morphological change allows the study of intratumoral heterogeneity *in vivo* tumor.

In summary, altering the calcium concentration in MCF-7 culture medium affects both the stability of the E-cadherins, which allows for the study of interstitial fluid pressure on varying intercellular adhesivity and the morphology, which allows for the study of interstitial fluid pressure on heterogenous MCF-7 tumor cells.

3.2.2 Enzyme Linked Immunosorbent Assay (ELISA) results

In ELISA test, the optical densities are measured by a microplate reader and it is directly related to the levels of protein concentration. The higher the protein concentration, the higher the optical densities. The level of E-cadherin expressions on MCF-7 cells are quantified by comparing the optical densities measured at 450nm wavelength against a standard curve (Figure 3-24) which have known protein concentrations. The standard curve is the best fit line of the optical densities of the known concentration subtracted with the background optical densities which is represented by the zero protein concentration (Table 6). The measured optical densities of the standards are listed (Table 6). The standards are prepared by serial dilution factor of 2.

Figure 3-42 Standard curve of known E-cadherin concentration and the corresponding optical densities.

Table 6 Mean optical densities for known protein concentration.

Protein concentration in standards (pg/mL)	O.D
0	0
156	0.125
313	0.215
625	0.311
1250	0.515
2500	1.123
5000	2.005
10000	2.564

Table 7 Optical densities of MCF-7 cell samples.

Calcium Concentration in Culture Medium	1.5mM	1.7mM	1.9mM
O.D	0.231	0.327	0.423

The optical densities of the samples which are MCF-7 cells cultured in 1.5mM, 1.7mM and 1.9mM calcium concentration culture medium less the background optical densities are 0.231, 0.327 and 0.423 respectively (Table 7). The optical densities of the samples fall within the range of the standards, so the concentration range of the standards are good. The optical densities of these 3 cellular aggregates are extrapolated to find the concentration of protein (E-cadherin) in the samples. The E-cadherin concentration of MCF-7 cells cultured in 1.5mM, 1.7mM and 1.9mM calcium concentration culture medium is 365pg/mL, 532.85pg/mL and 805.24pg/mL respectively.

The E-cadherin concentration of MCF-7 cells cultured in 1.5mM calcium concentration culture medium is ~1.46 and ~2.2 times lower than the E-cadherin concentration of MCF-7 cells

cultured in 1.7mM and 1.9mM calcium concentration culture medium respectively. This result is expected as the MCF-7 cells are cultured in different calcium concentration and E-cadherins being calcium dependent adhesion molecules are affected by the calcium concentration [89] [90]. With lower calcium concentration in the 1.5mM calcium concentration culture medium, the MCF-7 cells express lower levels of E-cadherins as compared to MCF-7 cells cultured in 1.7mM and 1.9mM calcium concentration culture medium [54].

This result shows that the alteration of calcium concentration in MCF-7 culture medium is a good way to affect the E-cadherin expression levels. With different E-cadherin levels on MCF-7 cells, the aggregates form from MCF-7 cells cultured in 1.5mM, 1.7mM and 1.9mM calcium concentration will have different adhesion levels. Together with the change in morphology of MCF-7 cells cultured in different calcium concentration and different adhesions due to different E-cadherin expression levels, the effects of interstitial fluid pressure on different intercellular adhesivity of MCF-7 to study the heterogeneity can be studied and the objective of this project can be met.

3.2.3 High loading rate ($1\mu\text{l}/\text{min}^2$) across cellular aggregate in microfluidic device coated with 10% Fibronectin

The pressure difference between the afferent and efferent ends of the cellular aggregate in the square chamber is able to give us an understanding on the intercellular adhesivity and most importantly, the effects of increasing interstitial flow across the cellular aggregate on the intercellular adhesivity of the cellular aggregate. The strength of MCF-7 cell-cell adhesion is dependent on the calcium concentration present in the culture medium. The calcium concentration affects the expression levels of E-cadherin and hence the strength of cell-cell adhesion.

The following is 1 flow experiment performed on cellular aggregate cultured in 1.5mM calcium concentration culture medium (Figure 3-25) to demonstrate the changes in instantaneous

Figure 3-43 Graph of pressure measured at afferent ends (Red) and efferent ends (Blue) of cellular aggregate cultured in 1.5mM calcium concentration culture medium.

pressure at the afferent and efferent ends of a cellular aggregate in the microfluidic device. The red dots and blue dots represent the instantaneous pressure at the afferent ends (start of cellular aggregate) and efferent ends (end of cellular aggregate) of 1.5mM cellular aggregate respectively in the square chamber (Figure 3-25).

For the flow experiment, high loading rate ($1\mu\text{l}/\text{min}^2$) is introduced at the afferent ends of the cellular aggregate. Cellular aggregates cultured in 1.5mM, 1.7mM and 1.9mM calcium concentration culture medium is used. Pressure is recorded at both the afferent and efferent ends of the cellular aggregate with a digital manometer. Instantaneous pressure at afferent and efferent ends are recorded at 1s interval. 5 flow experiment were conducted for each calcium concentration culture medium.

During the flow experiment, DMEM is first introduced, the pressure at the afferent ends (red data points) of the cellular aggregate in the square chamber increases steadily. The pressure at the efferent ends (blue data points) of the cellular aggregate in the square chamber remains relatively constant for the first 3 minutes (Figure 3-25). DMEM with fluorescent dye can be seen seeping through the cellular aggregate (Figure 3-26 (A)). After 3 minutes, culture medium with fluorescent dye can be seen at the efferent ends of the cellular aggregate (Figure 3-26 (B)) and this is when the pressure at the efferent end start to increase. This is reflected in the gradual increase of pressure reading at the efferent ends of the cellular aggregate (Figure 3-25). The rate of increase of pressure is higher at the afferent ends than the efferent ends of the cellular aggregate in the square chamber. This pressure difference drives flow through the cellular aggregate.

Fractures in the cellular aggregate are formed (Figure 3-26(C)) when the cellular aggregate can no longer withstand the pressure buildup at the afferent end. The fractures within the cellular aggregate decreases the hydraulic resistance and increases the hydraulic permeability through the cellular aggregate, increasing fluid flow through the cellular aggregate. This is reflected in the sudden pressure drop in the afferent ends (red data points) of the cellular aggregate (Figure 3-25). Immediately after fractures are formed, masses of the cellular aggregate dislodge and flows towards the reservoir of the device (Figure 3-26(D)). This causes the pressure to increase at the efferent ends (blue data points) of the cellular aggregate. This is transient as the mass is carried with the flow to the reservoir, returning the pressure at the efferent ends to its normal. The masses of dislodged cells mimic the detachment of cancer cells from primary tumor and are carried with the fluid flow to the reservoir. This significantly reduces the pressure at the afferent ends of the cellular aggregate.

Figure 3-46 Cellular aggregate cultured in 1.5mM DMEM in square chamber of microfluidics device. (A) Cellular aggregate adheres to fibronectin coating on the walls of the microfluidic device. (B) DMEM mixed with FITC is introduced to the cellular aggregate at an increasing flow rate of $1\mu\text{l}/\text{min}^2$. Mixture of fluid creeps through the porous cellular aggregate. (C) Fractures caused by the increasing flow appears within the cellular aggregate. Single/multi cells detach from aggregate slowly. (D) Big mass of cellular aggregate detaches completely from the square chamber.

The pressure difference across the cellular aggregate that is cultured in 1.5mM calcium concentration culture medium is derived from the difference between the mean afferent and efferent ends of the cellular aggregate. The pressure difference (blue data points) is plotted against the instantaneous flow velocity that is injected from the syringe pump into the microfluidic device (red data points) to compare and understand the effects of increasing interstitial flow across the cellular aggregate on the intercellular adhesivity (Figure 3-27).

Figure 3-49 Graph of pressure difference between afferent and efferent ends of cellular aggregate cultured in 1.5mM calcium concentration culture medium.

3.2.4 Comparison between 1.5mM, 1.7mM and 1.9mM under high loading rate ($1\mu\text{l}/\text{min}^2$) in microfluidic device coated with 10% Fibronectin

To understand the effects of increasing interstitial flow across the cellular aggregate on the intercellular adhesivity of the cellular aggregate, there is a need to compare the pressure difference across the cellular aggregate cultured in 1.5mM, 1.7mM and 1.9mM calcium concentration culture medium. The flow experiment performed above on 1.5mM cellular aggregate is carried out for 1.7mM and 1.9mM cellular aggregate. The mean of 5 successful runs of each cellular aggregate is used for the following comparison.

Figure 3-51 Comparison of Mean Critical Fracture Pressure across cells cultured in 1.5mM, 1.7mM, 1.9mM calcium concentration medium under high loading rate ($1\mu\text{l}/\text{min}^2$).

The mean critical pressure before fracturing for cellular aggregate cultured in 1.5mM, 1.7mM and 1.9mM calcium concentration culture medium is 321 Pa, 510.2 Pa and 1036 Pa respectively (Figure 3-28). These values represent the mean critical pressure at which the cellular aggregates fractures and masses of cells dislodged. The coefficients of variance are 0.192, 0.075 and 0.136 respectively.

At high loading rate of $1\mu\text{l}/\text{min}^2$, cellular aggregate that are cultured in culture medium with higher calcium concentration ($1.9\text{mM} > 1.7\text{mM} > 1.5\text{mM}$) which expresses higher expression levels of E-cadherin fractures at a higher mean critical fracture pressure as compared to cellular aggregate that are cultured in culture medium with lower calcium concentration. This is reflected in the results presented in the figure above (Figure 3-28). The pressure difference between the afferent and efferent ends of the cellular aggregate before cellular aggregate in the square chamber fractures is significantly higher for cellular aggregate cultured in 1.9mM calcium concentration than 1.7mM and then 1.5mM. This result shows that with higher expression levels of E-cadherin, which translates to stronger cell-cell adhesivity, the higher the ability to withstand pressure difference driven by fluid flow.

This translates to *in vivo* tumor with morphology that expresses higher E-cadherin levels or stronger cell-cell adhesivity being able to withstand higher pressure gradient between tumor core and outside of tumor before fractures occur and masses of cells dislodged kickstarting the metastatic cascade.

3.2.5 Comparison between 1.5mM, 1.7mM and 1.9mM under low loading rate ($0.5\mu\text{l}/\text{min}^2$) in microfluidic device coated with 10% Fibronectin

Flow experiment on 1.5mM, 1.7mM and 1.9mM cellular aggregate with high loading rate ($1\mu\text{l}/\text{min}^2$) demonstrated that with stronger cell-cell adhesivity, the higher the ability to withstand pressure buildup and hence the cellular aggregate fractures at a higher mean critical fracture pressure.

Figure 3-54 Comparison of Mean Critical Fracture Pressure across cells cultured in 1.5mM, 1.7mM, 1.9mM calcium concentration medium under high loading rate ($1\mu\text{l}/\text{min}^2$).

Next, 1.5mM, 1.7mM and 1.9mM cellular aggregates are subjected to low loading rate flow ($0.5\mu\text{l}/\text{min}^2$). The decrease in loading rate simulates the lower pressure driven interstitial flow through the tumor due to a slower increase in pressure gradient across the tumor in our body.

The mean critical pressure before fracturing for cellular aggregates cultured in 1.5mM, 1.7mM and 1.9mM calcium concentration culture medium under low loading rate is 465.2 Pa, 472.2 Pa and 489.8 Pa respectively (Figure 3-29). The coefficients of variance are 0.090, 0.115 and 0.135 respectively.

The mean critical fracture pressure for cellular aggregates cultured in all calcium concentration culture medium under low loading rate do not differ significantly (Figure 3-29). The change in cell-cell adhesivity do not affect the mean critical fracture pressure under low loading rate. This means that under low loading rate, strengths of cell-cell adhesions are insensitive to the pressure gradient across the cellular aggregate.

These results when translated to *in vivo* means that if the pressure buildup within the tumor core is slow, causing the pressure driven interstitial flow to be increasing at a slow rate, the differences in strength of cell-cell adhesions do not play a significant role in affecting the fracture pressure. This result is in contrast to cellular aggregate under high loading rate where the strength of cell-cell adhesions affects the mean critical fracture pressure.

3.2.6 Comparison between high ($1\mu\text{l}/\text{min}^2$) and low loading rate ($0.5\mu\text{l}/\text{min}^2$) in microfluidic device coated with 10% Fibronectin

As mentioned above, the cellular aggregates cultured under 1.5mM, 1.7mM and 1.9mM calcium concentration culture medium represents low, medium and high cell-cell adhesions respectively and the varying strengths of cell-cell adhesions affects the mean critical fracture pressure under high loading rate but not at low loading rate.

Figure 3-57 Graph of Mean critical fracture pressure (Pa) against cellular aggregates cultured in 1.9mM, 1.7mM and 1.5mM calcium concentration culture medium. (A) Cellular aggregates undergo high loading rate ($1\mu\text{l}/\text{min}^2$). (B) Cellular aggregates undergo low loading ($0.5\mu\text{l}/\text{min}^2$)

Another difference that can be observed is that the mean critical fracture pressure across cellular aggregates cultured in 1.9mM and 1.7mM calcium concentration culture medium fractured at a lower mean critical fracture pressure under low loading rate as compared to high loading rate (Figure 3-30) but there is no significant difference in mean critical fracture pressure for cellular aggregate cultured in 1.5mM calcium concentration culture medium.

When this translates to *in vivo*, it means that for every different MCF-7 cell-cell adhesions, when the pressure buildup within the tumor is slow (Low loading rate) creating a lower rate of pressure driven interstitial flow, the MCF-7 tumor fractures at a lower mean critical fracture pressure. However, when the pressure buildup within the tumor is very fast (High loading rate), creating a high rate of pressure driven interstitial flow through the tumor, the stronger the cell-cell adhesions, the higher the pressure the tumor can withstand within the tumor core, maintaining a larger pressure gradient across tumor before fracturing.

3.2.7 Comparison between 10%, 20% and 30% Fibronectin under low loading rate ($0.5\mu\text{l}/\text{min}^2$) across 1.9mM cellular aggregate

In vivo tumor is surrounded by extracellular matrix (ECM). To investigate the biophysics of interstitial flow causing tumor cells to detach, extracellular matrix has to be considered as well. In the flow experiment, fibronectin coating on the microfluidic device acts as ECM, holds the cellular aggregate in the square chamber by creating an adhesive between fibronectin and periphery cells of the cellular aggregate. Fibronectin is a major component of ECM as well.

Figure 3-59 Comparison of mean critical fracture pressure across 1.9mM cellular aggregate in microfluidic device with 10%, 20% and 30% fibronectin concentration. The fibronectin concentration represents the strength of cell-ECM adhesions.

To study the role ECM plays in the detachment of tumor cells due to interstitial fluid flow driven by pressure, the cell-ECM adhesion strength is investigated. This is done by varying the fibronectin concentration which represents a major component in ECM. The fibronectin concentration is varied at 10%, 20% and 30% when introduced into microfluidic device to be

coated. The higher the percentage of fibronectin, the more the ECM and hence stronger cell-ECM adhesions.

Low loading rate ($0.5\mu\text{l}/\text{min}^2$) fluid flow is applied across cellular aggregate cultured in 1.9mM calcium concentration culture medium. The mean critical fracture pressure of 5 successful runs for each fibronectin concentration is shown (Figure 3-31).

The results show that with higher fibronectin concentration, the higher mean critical fracture pressure. This means that when the fibronectin concentration is high, the stronger the cell-ECM adhesion, the more the cellular aggregate is able to withstand pressure at the afferent end before fracturing.

This translates to *in vivo* tumor having more ECM around the tumor, the stronger the cell-ECM strength, the higher pressure buildup the tumor is able to withstand within the tumor core before fractures occur and dislodges at a higher mean critical fracture pressure to kickstart the metastatic cascade.

3.3 Summary

In this section of my work, I varied cell-cell adhesions in MCF-7 cells to mimics the *in vivo* tumor heterogeneity by altering the calcium concentration in the culture medium during culturing and varied the cell-ECM adhesions between MCF-7 cells and fibronectin to study the mean critical pressure to fracture the cellular aggregate using a previously designed microfluidic device.

This study shows that under high loading rate ($1\mu\text{l}/\text{min}^2$), mean critical fracture pressure of MCF-7 cellular aggregate is affected by E-cadherin expression level. With a change in E-cadherin expression levels in MCF-7 cells which affects the cell-cell adhesion, the mean critical pressure to fracture the cellular aggregate is affected. When E-cadherin expression level is highest (1.9mM), cell-cell adhesion is the strongest and the mean critical fracture pressure is the highest. This means that the cellular aggregate in the microfluidic device is able to withstand a large pressure difference between the afferent and efferent ends of the cellular aggregate before fracturing. When translated to *in vivo* tumor, tumor with higher E-cadherin expression levels can withstand a larger pressure gradient across tumor before fracturing and masses of cells dislodged to kickstart the metastasis cascade. The lower the calcium concentration in culture medium, the lower the expression levels of E-cadherin, the lower the cell-cell adhesion strength between MCF-7 cells and in turn would fracture under a smaller mean critical pressure.

This study also shows that under low loading rate ($0.5\mu\text{l}/\text{min}^2$), the mean critical fracture pressure is not affected by E-cadherin expression levels. There are no significant differences in mean critical fracture pressure for all cellular aggregate that was used in the flow experiment (1.5mM, 1.7mM and 1.9mM). Under low loading rate, the change in E-cadherin expression

levels affects the cell-cell adhesions but the mean critical fracture pressure remains relatively constant. This means that even with varying cell-cell adhesions, cellular aggregates in the microfluidic device is able to withstand relatively constant pressure difference before fracturing. When translated to *in vivo* tumor, tumor with varying E-cadherin expression levels can withstand same pressure gradient across tumor before fracturing.

This study also shows that under low loading rate ($0.5\mu\text{l}/\text{min}^2$), the mean critical fracture pressure is affected by fibronectin concentration. The higher the fibronectin concentration the stronger the cell-ECM adhesions. Under the same flow conditions ($0.5\mu\text{l}/\text{min}^2$) and same E-cadherin expression, the mean critical fracture pressure is higher when the fibronectin concentration is higher (30% vs 20% vs 10%). When translated to *in vivo* tumor, the tumor is able to withstand a larger pressure gradient before fracturing and masses of cells dislodging and kick start the metastatic cascade when there are more ECM in the tumor microenvironment and hence stronger cell-ECM adhesions.

Further experimental runs can be conducted on MCF-7 cells cultured in further reduced calcium concentration culture medium under the same flow conditions, both high and low loading rate. With more data of mean critical fracture pressure on MCF-7 cells cultured in reduced calcium concentration culture medium and hence reduced expression levels of E-cadherin, a data base can be generated for clinicians who detect breast cancer patients with MCF-7s to use to determine patient's prognosis.

More experimental runs will also be conducted using higher fibronectin concentration to coat the microfluidic device. This result will help in finding the saturation concentration of fibronectin.

4 Dielectrophoresis to sort viable and unviable breast cancer cells (MCF-7)

In the previous chapter, the focus is on different E-cadherins expression which affects the cell-cell adhesion strength of a cellular aggregate and the effect of increasing flow rate of $1\mu\text{l}/\text{min}^2$ have on different cell-cell adhesion strength within MCF-7 cellular aggregate. Along the same thought on studying cell-cell adhesion strength, studying cell-cell adhesion for single cell is equally important. In this chapter, I demonstrated the use of dielectrophoresis (DEP) to manipulate MCF-7 to form a chain of cells so that cell-cell adhesion strength of MCF-7 can be studied in the future.

4.1 Materials and Methods

4.1.1 Fabrication of DEP microfluidic chip

The microelectrodes were fabricated with standard cleanroom lithography techniques. First, the design of the microelectrodes was patterned onto a 4" Borofloat 33 glass wafer with positive photoresist (AZ9260, Microchemicals GmbH). Subsequently, a layer of 10 nm of chromium and 200 nm of gold was magnetron sputtered onto the glass wafer. The microelectrodes have a gap of $100\mu\text{m}$ for breast cancer cell MCF-7 experiments. An open reservoir design was used for the breast cancer cell experiment for the ease of pipetting from cell culture. Furthermore, the open reservoir design adopted for the cancer cell experiment is to achieve no flow condition, where the movement of the cells are induced only by dielectrophoresis and gravitational force.

4.1.2 Cell Culture – MCF-7

The cells used for the experiment were the Michigan Cancer Foundation-7 (MCF-7) purchased from American Type Culture Collection (ATCC HTB-22). MCF-7 has a doubling time of 29

hours. First, MCF-7 cells are subcultured into thermo fisher T-75 flask at a seeding density of 10^6 cells. Culturing media which consists of 89% DMEM, 10% FBS and 1% penicillin is replaced every 3 days. After 1 week, the MCF-7 cells reach 70% confluency and this is the time where MCF-7 can be used for experiment. To start the experiment, MCF-7 cells which are adhering as a mono layer on the culturing flask are washed thrice with Phosphate Buffer Solution (PBS). After the third wash, 3ml of trypsin-EDTA (0.25%) is added and incubated in 5% CO₂ for 4 minutes. Subsequently, 5ml of culture media is added into the culturing flask to neutralize the effect of trypsin-EDTA (0.25%). The mixture of trypsin-EDTA (0.25%) and DMEM is transferred to a centrifuge tube and centrifuged at 1,500 rpm for 7 minutes and resuspended in DI-water.

4.1.3 Experimental Setup

500 μ L of breast cancer cells were pipetted directly on the microelectrodes surface. DEP force is applied to through applying a sinusoidal voltage of 3 – 5 V peak-to-peak at frequencies ranging from 200 Hz to 20 MHz via a function generator (SDG2122X, Siglent).

4.2 Results and Discussion

4.2.1 DEP of Breast Cancer cell

MCF-7 cells experiencing DEP forces in the microfluidic device presents similar behavior to bacteria cells. Under positive DEP forces (1kHz-50 kHz), the MCF-7 cells are attracted towards the parallel gold microelectrode. On the other hand, under negative DEP forces (500kHz-20MHz), the MCF-7 cells repel from the parallel gold microelectrode and form a chain in the mid-axis of the electrode's gap. The dielectrophoresis behaviors of MCF-7 cells under applied electric field of 5 V_{pp} at frequencies of 1 kHz, 10 kHz, 50 kHz, 500 kHz, 1 MHz, and 20 MHz is shown in Figure 4-1(A). The time evolution of cell chain formation under negative DEP at 5 V_{pp} and 20 MHz for 50 minutes at every 10 minutes intervals is shown in Figure 4-1(B).

This behavior is the result of interaction between induced dipoles of cells and the gradient of the electric field from the parallel gold microelectrode. MCF-7 cells under negative DEP forces are repelled and move the furthest away from the parallel gold microelectrode and they form a chain of cells. The gradient of the electric field is steepest along the microelectrode edges. In negative DEP, the cells are repelled from these edges and migrate towards the middle of the gap between electrodes, where the local field gradient is shallowest (Figure 4-2).

Figure 4-1 (A) MCF-7 cells under DEP force of 5 V_{pp} at frequencies ranging from 1 kHz to 20 MHz. From 1 kHz-50 kHz, the DEP is positive and the MCF-7 cells are attracted towards the parallel microelectrode. From 500 kHz-20 MHz, the DEP is negative and the MCF-7 cells are repelled from the parallel electrodes. (B) MCF-7 cell chain formation at 5 V_{pp} and 20 MHz at every 10 minutes intervals. The DEP is negative. The MCF-7 cells are induced by the negative DEP and move towards the centre, away from the parallel microelectrode.

Figure 4-2 The surface contour plot represents the electric potential (V/m) (A) The x-y plane has height 0.5 μm . The electrode is 1 μm . The field gradient has significant difference only around 20 μm of the electrode surfaces. Other regions shows mostly relatively constant field gradient. Under negative DEP, cells are observed to be repelled from the microelectrodes. (B) The plane shown is taken at x-z mid-plane of the electrode length. The variation of electric potential gradient with channel depth is observed in regions directly above electrode corners, from which the electrode-trapping effect of positive DEP can be explained.

MCF-7 cells tend to adhere to each other once the chain of cells is formed. This is because MCF-7 cells possess intercellular structures that holds cells together. MCF-7, which is of epithelial cell origin, has an affinity to adhere to each other through cell adhesion molecules (CAMs) and transmembrane proteins (such as the E-cadherin, N-cadherin, and integrins) [96]. The proteins form homotypic binding to hold cells together.

In recent times, many researches have applied DEP forces on cells under flow in microfluidic devices for cell separation and immobilization of cells without the need of any labeling techniques [97] [98]. This allows single viable cells to be studied. In this current setup, the application of DEP forces alone is able to form a chain of adhering cells, parallel to the microelectrode (Figure 4-1(B)). The chain of adhering cells can be collected to be used in the study of single cell-cell adhering strength from the setup. This opens up opportunities to study intercellular interactions. The DEP setup gives the ability to study and quantify intercellular adhesion strength between MCF-7 cells. This would allow us to better understand the biophysics of MCF-7 in a tumor environment.

The sign real part of the Clausius-Mossotti (CM) factor, $\text{Re}[f_{CM}]$ determines whether the DEP force of a cell is an attraction (positive DEP) or repulsion force (negative DEP). The $\text{Re}[f_{CM}]$ for MCF-7 cells in DI-water, PBS and DMEM suspension for frequency ranging from 1 Hz to 10^{12} Hz are computed with MyDEP software [99]. From the CM factor plot (Figure 4-3), we observed that when PBS or DMEM are used as the suspension buffer, the real part of CM factor for MCF-7 is always negative regardless of the applied frequency. In other words, with PBS and DMEM only negative dielectrophoresis is possible. On the other hand, MCF-7 cells were able to experience both the positive and negative DEP in DI water. This would be important for cell

manipulation application using DEP. Therefore, in this work, DI water was chosen for the MCF-7 cells to demonstrate both positive DEP and negative DEP. Nonetheless, due to the difference in osmotic pressure, immersion of MCF-7 cell in DI water is not ideal for the cell survival. For the duration of the experiment, the cells morphology remains largely unchanged. However, it should be noted that after the cells started to lysed after more than an hour in DI water suspension, leaving only cell debris on the electrodes.

Figure 4-5 MyDEP software computed the CM factor's real part for MCF-7 cells in DI water, PBS and DMEM. For both the PBS and DMEM, only negative DEP can be applied regardless of the applied frequency from 1 Hz to 1012 Hz. On the other hand, MCF-7 cells in DI water, the cells could experience both positive and negative DEP, with a cross-over frequency of 236 MHz.

4.3 Summary

In summary, the setup allows DEP to induce the formation of human breast cancer cell (MCF-7) chain under negative DEP force. More experimental runs will be conducted on MCF-7 cells cultured in different calcium concentration culture medium to compare the effect of DEP on them. After which, the MCF-7 cells can be collected, and single cell-cell adhesion strength can be further investigated.

5 Conclusion

In this project, microfluidic device is used to study the effects of interstitial flow on detachment of MCF-7 cells from cellular aggregate with varying cell-cell adhesion strength and cell-ECM adhesion strength. Results show that under high loading rate flow, the stronger the cell-cell adhesions of MCF-7 cells in the cellular aggregate, the higher the mean critical fracture pressure before fracture occurs and detachment of MCF-7 cells from cellular aggregate. Under low loading rate flow, the effects of varying cell-cell adhesions on the mean critical fracture pressure are insignificant.

While varying the fibronectin concentration to simulate changes in cell-ECM adhesion strength, results show that with an increase in cell-ECM adhesion strength cause an increase in mean critical fracture pressure. These results show that both the cell-cell and cell-ECM adhesions do play a significant role in affecting the tumor's ability to withstand pressure gradient across tumor to prevent fracture and hence masses of cells from dislodging, kickstarting the metastatic cascade.

Dielectrophoresis (DEP) is also employed on MCF-7 cells. This allows for the sorting of viable MCF-7 cells. At $5V_{pp}$ and at frequency between 10kHz to 50kHz, positive DEP force attracts MCF-7 cells to the electrodes. At $5V_{pp}$ and at frequency between 500kHz to 20MHz, negative DEP force repels MCF-7 cells away from the parallel electrode to form MCF-7 chains. At 20MHz and 50mins, MCF-7 chains form at the center between the parallel electrodes. This technique provides a potential method to collect individual chains of MCF-7 cells by introducing flow after the chain is formed so that these cells can be collected, and cell-cell adhesions of single cell can be studied in addition to the cell-cell adhesions from a cellular aggregate.

This project gives us insights into the role cell-cell and cell-ECM adhesions play in MCF-7 cells detachment under interstitial fluid pressure flow. This project also shows the potential of using DEP to collect individual viable MCF-7 pearl chains and allow us to study cell-cell adhesion strength of individual cells.

6 Future Works

6.1 Microfluidics modeling of interstitial flow through a tumor

Moving forward from the data collected and understanding of the effects of interstitial fluid flow on varying cell-cell adhesion strength, more can be done. I propose to further vary the calcium concentration for the culturing of MCF-7 cells. This includes both increasing and decreasing calcium concentration for the culturing of MCF-7 cells. This can affect the E-cadherin expression levels and hence the cell-cell adhesion strength. The same loading rate ($1\mu\text{l}/\text{min}^2$) can be applied, and the mean critical fracture pressure is recorded and tabulated. With more experimental runs done on cellular aggregates with a range of E-cadherin expression, a benchmark can be created. This benchmark can potentially be used by clinicians to predict the prognosis of patients with breast cancer that presents MCF-7 cell lines.

Besides varying the cell-cell adhesions, I also propose to further increase the fibronectin concentration for the coating of the microfluidic device. This increase would allow for better understanding of the effects of interstitial flow on cell-extracellular matrix strength.

Combining the study of the effects of interstitial flow on cell-cell adhesions and cell-ECM adhesions, there will be a more complete understanding as a whole for both future researchers interested in studying the biophysical aspects with respects to interstitial flow and to future clinicians.

6.2 Study of tumor cell under dielectrophoresis

From chapter 4 results, negative DEP repels MCF-7 cells away from the electrodes to form a chain. Moving forward, I propose to use the same DEP setup on MCF-7 cells that are cultured in different calcium concentration. This potentially allows for a few studies to be done. Firstly, this allows us to record any differences in formation of MCF-7 chains that are cultured in different calcium concentration. Secondly, this also allows us to be able to form MCF-7 cell chains that expresses difference levels of E-cadherin and hence possess different cell-cell adhesions.

I also propose to incorporate flow to the current DEP setup after the MCF-7 cell chains are formed to collect these chains downstream so that individual cell-cell adhesion strengths can be investigated. This is important because unlike the previous microfluidic setup which investigates the mean critical fracture pressure of a cellular aggregate with varying cell-cell adhesion strength, MCF-7 cell chains form with the DEP setup is an individual cell-cell adhesion. The study of individual cell-cell adhesion strength, which is cultured in varying calcium concentration, coupled with the previous study which involves cell-cell adhesion strength of MCF-7 within a cellular aggregate would give a more complete understanding.

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8 Appendix

8.1 Appendix A – Photo-lithography fabrication process with SU-8 2100 on a silicon wafer

1) Clean wafer with acetone and isopropanol. Rinse it with distilled water. Use nitrogen blower to dry the wafer surface.

2) Heat wafer on a hot plate at 110 °C for 7 minutes.

3) Spin coat SU-8 onto the wafer

- First spin: 500 rpm, at 100 rpm s⁻¹ for 5-10 seconds

- Final spin: 2000 rpm at 300 rpm s⁻¹ for 30 seconds

4) Prebake the wafer at 65 °C for 10 minutes and then bake at 95 °C for 25 minutes. After baking, cool wafer slowly to room temperature.

5) Align mask with the wafer by Mask Aligner machine

6) UV exposure based on MicroChem manual with the contact type as hard.

7) After exposure, bake the wafer at 65 °C for 10 minutes then continue baking at 95 °C for 15 minutes on the hot plate.

8) Develop the wafer 10 minutes.

9) Rinse the wafer with IPA and DI water, and then dry it by nitrogen blower or by wafer dryer machine.

8.2 Appendix B – Making of Device

Polydimethylsiloxane - PDMS

- 1) Determine the necessary volume of PDMS needed each time
- 2) Mix the PDMS monomer with the curing agent at ratio 10:1.
- 3) Degas PDMS mixture to remove bubbles introduced during mixing
- 4) Gently pour the PDMS mixture onto wafer with design. Make sure to remove any bubbles with pipette
- 5) Bake PDMS in an oven at 60 °C for 2-4 hours.
- 6) Cut the cured PDMS in patterned areas.
- 7) Punch the holes for the PDMS chips by using Harris Unicore puncher.
- 8) Soak the PDMS chips into distilled water for 24 hours to deionize them.

Plasma bonding

- 1) Clean glass slides with acetone and isopropanol and finally rinse with DI water
- 2) Apply air plasma treatment with power 30 W for 30 to 45 seconds to activate the surfaces of PDMS and glass slides.
- 3) Press PDMS gently against glass slides

4) Bake bonded device in oven at 80⁰ C for 2 hours.

8.3 Appendix C - Cell culture procedure

- 1) Remove culture medium inside the flask.
- 2) Rinse monolayer cells twice with 10 ml PBS
- 3) Add 3 ml of 0.25% (w/v) Trypsin - 0.53 mM EDTA solution and put flask into 37 °C incubator for 4 minutes.
- 4) Check cells under an inverted microscope to ensure cell detaches from flask surface. Refrain from shaking or tapping on flask to detach cells.
- 5) Add 5 ml of culture medium to deactivate trypsin
- 6) Pipette trypsinized cells into a centrifuge tube.
- 7) Centrifuged suspension of cells and medium at 1500 rpm for 7 minutes.
- 8) Remove the supernatant.
- 9) Re-suspend cells with 2 ml of fresh culture medium.
- 10) Count cells using hemocytometer:
- 11) Add appropriate volume of cell suspension to a new culture flask with 12ml of culture medium
- 12) Put the flask into the incubator at 37 °C and 5% CO₂ atmosphere.

8.4 Appendix D – Cellular Aggregate production

- 1) When reaching 70% confluency, cells in the culture flask are treated with 0.25% (w/v) Trypsin - 0.53 mM EDTA and then washed with culture medium to deactivate trypsin.
- 2) Centrifuged suspension of cells and medium at 1500 rpm for 7 minutes.
- 3) Cells are re-suspended in 2 ml fresh culture medium and transferred to a 25-ml Erlenmeyer flask containing 5 ml CO₂-equilibrated culture medium at a concentration of 2×10^5 cell ml⁻¹.
- 3) Place the Erlenmeyer flask inside an incubated orbital shaker at 37 °C spinning at 75 rpm for 24 hours.

8.5 Appendix E - Moving cellular aggregate into square chamber

- 1) The heating stage of the microscope, where the microfluidic device is placed inside, need to be off. The temperature of the device is at room temperature. All the microchannels need to be pre-filled with CO₂-equilibrated culture medium.
- 2) Two scissor clamps (with flat jaws) are employed to clamp manometer tubing at channel 2 and 4.
- 3) Unplug the tubing of Pump 2 in channel 3 inlet off the PDMS device
- 4) Pipette selected cellular aggregate with diameter of 400-500 μm and introduce to channel 3
Then re-plug the tubing of Pump 2 to channel 3 inlet.
- 5) Turn on the live imaging of the microscope to observe real-time aggregate movement. Control syringe pump to make sure cellular aggregate not moving too fast.
- 6) Start by applying between 100-200 $\mu\text{l min}^{-1}$ flow rate from Pump 2 to make the cellular aggregate move to the junction between the inlet and the microchannel 3. Stop syringe pump when the cellular aggregate partially enters the channel. Reduce flow rate appropriately to ensure cellular aggregate move through channel 3 slowly so as not to break the cellular aggregate at the square chamber.
- 7) Stop the pump when cellular aggregate enters square
- 9) Release the clamps at the two manometer tubings.

8.6 Appendix F – ELISA Test

PBS

- Mix PBS 10X @ pH 7.2 with DI water

Wash Buffer

1. For each runs of 16 samples and 14 standards plus 2 extra wells for IC Diluent 15, there should be 384 washes in total. (Make enough for 375 washes) Account for 3 washes for plate preparation and 3 washes after the incubation of sample/standard, 3 washes after adding detection antibody and incubation and 3 washes for after adding the Strep-HRP. A total of 12 washes for 32 wells.
2. Each wash requires 400uL of wash buffer, so the total wash buffer required is 153.6mL. I would make 200mL.
3. Make 10%Tween 20 first by mixing 9mL of DI water with 1mL of Tween 20. Use a cylinder and put 9mL of DI water then add tween 20 until reaches 10mL.
4. Mix 1mL of 10% Tween 20 with 199mLmL of PBS 1X

BSA Stock solution

1. Prepare 10% BSA stock.
2. Mix 10g of BSA into 100mL DI water. Can use the 0.2um pore size syringe filter

Block Buffer

1. Block Buffer is required during plate preparation
2. 300uL is needed for each well.
3. Each experimental run, I will use 32 wells. A total of 9.6mL is needed.
4. I will prepare 12mL of block buffer
5. Mix 1.2mL of 10% BSA stock, 60uL of 10% sodium Azide and 1.2mL of 10x PBS with 9.54mL of DI water

IC Diluent 1

1. Prepare 10mL
2. Mix 1mL of 10% BSA with 1mL of 10x PBS plus 8mL DI water and then filter with 0.2um Millipore filter

IC Diluent 15

1. Make 100mL of IC Diluent 15
2. 5M of NaCl
3. Prepare 10ml of 10% stock SDS by dissolving 1g of SDS in 10mL in water bath (55degree).
4. Mix 5ml of 1M Tris (Axil Scientific), 3ml of 5M NaCl, 10ml of 10% NP-40 alternative, 1ml of 10% SDS and 500mg of Sodium deoxycholate with 81mL of DI water

Lysis buffer

1. Cell density is @5mil cells per flask, so with that and the protocol stated, I would need 500ul of cell lysis buffer for each flask.
2. To test for 1.9mM, 1.5mM and 1.2mM cells, I will need a total of 1.5mL of lysis buffer.
3. To make 12mL of lysis buffer
4. Use 0.5M EDTA Stock from Dr Chan
5. Mix 0.021g of NaF with 1mL DI water to get 0.5M
6. Mix 0.0871g of PMSF with 5mL 100% ethanol to get 0.1M
7. Mix 1.3295g of sodium pyrophosphate with 5mL of DI water to get 1M
8. Mix 1.839g of Activated sodium orthovanadate with 10mL DI water to get 1 M
9. Mix 600ul of 1M Tris, 1.2mL of 10% NP-40 alternative, 30mg of sodium deoxycholate, 360ul of 5M NaCl, 120uL of 0.5M EDTA. Store this in 4deg fridge.
10. Prepare aprotonin by dissolving 10mg in 1mL of DI water to form 10mg/ml
11. Prepare Leupeptin by dissolving 25mg in 1ml of DI water to form 10mg/ml
12. Prepare Pepstatin by dissolving 10mg in 10ml of ethanol and put it in water bath to form 1mg/ml
13. Mix in to step 9 soln, 120uL of 0.5M NaF, 1.2uL of PMSF, 24uL of 1M sodium pyrophosphate, 12uL of activated sodium orthovanadate, 12ul of 10mg/ml leupeptin, 120ul of 10mg/ml of pepstatin, 3.6ul of 10mg/ml of aprotinin with 10mL of PBS

Cell preparation

1. After trypsinizing and spinning, rinse cells with PBS twice. Centrifuge to remove PBS
2. For 5 mil cells per flask, put in 500ul of lysis buffer and resuspend (vortex for 10-30s).
Put on ice for 15 mins
3. Collect the cell lysate in a 1.5ml or 2ml microcentrifuge tube before centrifuge
4. Centrifuge at 10000xg for 5 mins and transfer supernatant to clean tube.
5. Can store some tubes @-30 for short term use later

Plate preparation

1. Done 1 day before experimental run
2. Dilute capture antibody by adding 36mL of PBS.
3. Only use 100ul x 32 wells.
4. Seal plate and incubate overnight at room temperature
5. Aspirate each well and wash with Wash Buffer, repeating the process two times for a total of 3 washes. Wash by filling each well with Wash Buffer (400 μ L)
6. Block plates by adding 300 μ L of Block Buffer to each well. Incubate at room temperature for 1-2 hours.
7. Repeat the aspiration/wash as in step 2. The plates are now ready for sample addition.

Assay Procedure

1. Add 100 μ L of sample or standard in IC Diluent #15 per well. Use IC Diluent #15 as the zero standard. Cover with a plate sealer and incubate 2 hours at room temperature.
Note: *A seven point standard curve using 2-fold serial dilutions and a high standard of 10,000 pg/mL is recommended.*
2. Repeat the aspiration/wash as in step 2 of the Plate Preparation. 3. Add 100 μ L of the diluted detection antibody to each well.
3. Cover with a new plate sealer and incubate 2 hours at room temperature.
4. Repeat the aspiration/wash as in step 2 of the Plate Preparation. 5. Add 100 μ L of the diluted Streptavidin-HRP A to each well.
5. Incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.
6. Repeat the aspiration/wash as in step 2 of the Plate Preparation.
7. Add 100 μ L of Substrate Solution to each well. Incubate for
8. 20 minutes at room temperature. Avoid placing the plate in direct light.
9. Add 50 μ L of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well immediately, using a microplate reader set to 450 nm with wavelength correction set to 540 nm.