ENS Paris Saclay
M2 PhysEnBio

MICROFLUIDICS FOR CANCER METASTASIS INVESTIGATION

Moein DERAKHSAHAN
moein.moein_derakhshan@ens-paris-saclay.fr

Encadrant : Sakina BENSALEM
sakina.bensalem@ens-paris-saclay.fr

29/01/2025

1 Introduction

Metastasis is the phenomena of migration of cancer cells. The process starts by a cancer cell breaking off from the primary tumor, traveling in the lymph or blood vessels, and eventually extravasating into a new tissue to grow and divide to form a new tumor in the secondary site. Only about 0.001–0.02% of cancer cells that enter circulation end up as a metastatic tumor, but the problem is once this secondary tumor is formed morbidity and mortality of patients increases significantly. Although the 5-year survival rates for localized cancers are relatively high, those for metastases are of 30% and have not improved significantly over the last 10 years**[1]**, making metastasis the major cause of death from cancer, as reported by the World Health Organization**[2]**. This shows the vitality of this matter to be investigated. Thus, understanding of what is cancer metastasis and how it is done in the cellular and molecular dimensions is essential for us to effectively combat cancer.

Significant efforts have been spent on investigating cancer metastases. Many of these studies focused on the causes of metastatic specificity. For example, breast cancer mainly metastasizes to bone, lung, brain, and liver tissues, whereas prostate cancer primarily metastasizes to bone **[3]**. This tendency suggests that cancer cells receive and respond to signals from the secondary site, leading to preferential migration. These signals might take the form of chemokines released from the secondary tumor site, mechanical properties of the environment that guide the migration of cancer cells, or interactions with other types of cells. Metastatic cancer cells were shown to adjust their migration mechanisms in response to these signals and more robustly than non-metastatic and normal cells **[4]**. It was also found that certain cancer drugs can lead to increased metastasis **[4]**. These factors highlight the need to investigate treatments designed specifically for metastasis. Currently, treatments for cancer and metastasis are often tested in vivo using mouse models in which cancer cells are injected orthotopically or into circulation. However, use of human cancer cells in mice requires shutting down the immunity system of mice, which renders the power of this approach limited because immune cells play an important role in metastasis. This might be the reason that many drugs tested in mice have failed to work in humans. Also, Observing and imaging of this procedure in vitro has its own limitations both control-wise and equipment-wise for 3D tracking of cells.

A few in vitro systems have been developed to predict individualized drug response for primary tumors, and these systems often involve isolation and culture of primary tumor cells with chemotherapy drugs. The growth of these tumor cells can be monitored as well as their gene expression and other measures. More sophisticated systems have used tumor spheroids and incorporated extracellular matrix (ECM) and other cell types. However, the response of cancer cells to drugs might differ because they encounter different environments during metastasis. Therefore, systems specifically for metastasis are direly needed.

The first conventional in vitro system used to study metastasis was the 2D wound-healing assay, which involves creation of a 'wound' gap in a cell monolayer and observation of cell migration to close the 'wound' gap**[5]**. Although simple to use, this approach fails to replicate the 3D environment and the signal gradients that are present in vivo. The study which I have been focused on during this report used this method too, but the main approach was not that.

An interdisciplinary approach involving biology and engineering science provides a promising opportunity. With an increasing understanding of the in vivo physical microenvironment, more researchers are utilizing an engineering strategy, especially microfluidics, to replicate such microenvironments found in vivo. This approach simplifies and re-establishes mechanical microenvironment on in vitro devices, enabling discoveries at the cellular and even molecular scale. Microfluidics has emerged as a powerful platform for study of cancer metastases and drug screening. Due to their micro-scaled structures, microfluidic devices require low numbers of cells and offer the potential for high-throughput screening. Microfluidic devices also allow for additional types of cells to co-exist in 3D while maintaining each cell population in their appropriate environment, which enables the intricate physiological environment during metastasis to be replicated.

Here, I will delve into a study which used microfluidic device to monitor the stiffness of brain cancer cells and compare it to benign cells to conclude mechanical properties of these cells which effect the metastasis of them**[6]**. But first, I will illustrate a big picture of microfluidic devices use in metastasis studies for different stages of this process in next section of this report.

2 Process of Metastasis

The progression of metastatic disease is a multifaceted and intricate process, typically encompassing a series of sequential stages : cancer cells invade the surrounding tissue (invasion), then enter the lymphatic and/or blood circulatory system (intravasation), travel through and survive the bloodstream (circulating), escape from the blood vessel (extravasation), and finally adapt and grow in a colonial environment (metastatic colony)(Figure 1). Therefor, a cancer cell has different challenges to overcome at every step of the metastasis, and we can monitor its behavior corresponding to various aspects of environmental situations. There has been many studies focusing on cancer cell extravasation and migration at the secondary site under (a) mechanical stimulation (subsection 'Microfluidic investigation of mechanical factors in cancer cell migration'), (b) biochemical factors (subsection 'Microfluidic investigation of biochemical signals in cancer cell invasion'), and (c) cells of the secondary site (section 'Microfluidic metastasis-on-a-chip models for investigation of cancer extravasation').

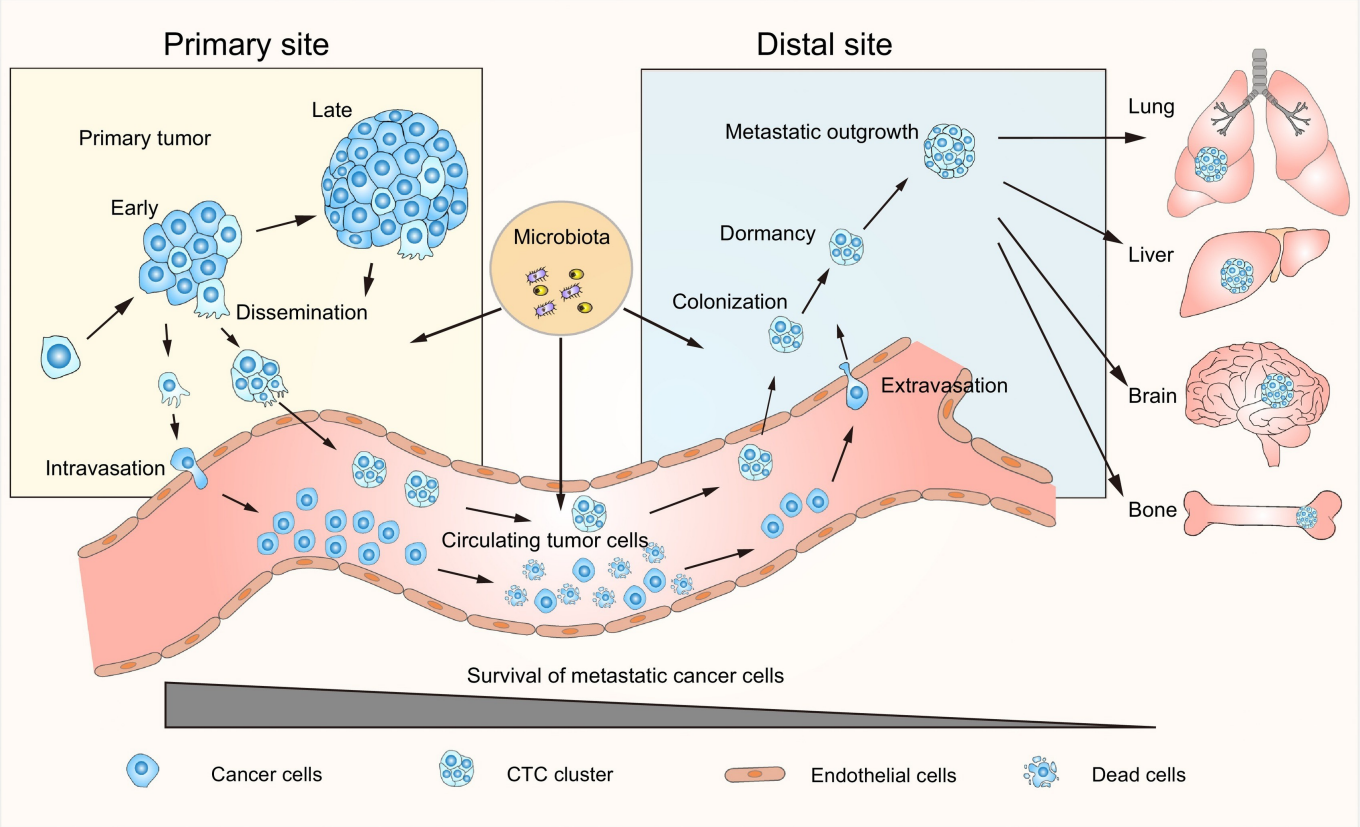

FIGURE 1 – Schematic view of metastasis' steps **[7]**.

2.1 Microfluidic investigation of mechanical factors in cancer cell migration

During the metastatic process, cancer cells encounter and respond to various mechanical stimuli from their environments. These cancer cells can convert mechanical signals into biochemical responses, influencing key biological processes such as survival, proliferation, and dissemination. In these kind of studies we attack the mechanical properties of migrating cancer cells and their environment. These studies focus both on the primary and secondary tumor sites. The focus is mainly on properties such as ECM stiffness, ECM viscoelasticity, flow shear stress, and extracellular fluid viscosity **[8]**. Figure 2 shows an example microfluidic device in which the authors tried to study the influence of environment confinement on cancer metastasis.

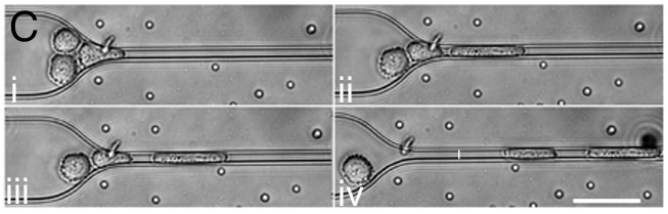

FIGURE 2 – Aggregates of cancer cells showing rearrangement and migration into confinement channels (time i to iv) **[9]**.

One of the interesting studies focusing on this aspect was the one done by Mak M and others, throughout which they developed a microfluidic device in which migrating cancer cells encountered a branch point leading to two channels, one $10\mu m$ wide and the other $3.3\mu m$ wide**[10]**. The two channels were configured in a circular (two branches splitting at the same angle) or semi-circular (smaller channel collinear with the original path leading to the branch point) pattern. Cancer cells were observed to either enter directly into one path or first extend into both channels. Approximately 90% of the cells entered the larger channel for circular branch points, whereas 68% did so for semi-circular branch points. Moreover, only 35% of cells treated with blebbistatin (inhibitor of non-muscle myosin II a) entered the larger channel at semicircular branch points, and Taxol significantly reduced the probability of cancer cells making a path decision and migrating further.

2.2 Microfluidic investigation of biochemical signals in cancer cell invasion

After cancer cells are arrested in the capillaries or slowed down in the sinusoid blood vessels, gradients of biochemical factors secreted by cells in the microenvironment might signal the tumor cells to extravasate out of the vessel. Cancer cell migration can be driven by various biochemical factors, such as epidermal growth factor, Hepatocyte growth factor, and vascular endothelial growth fac-

tor. Early models of microfluidic devices for these kind of studies have shown that cancer cells change the shape and matrix of the surroundings, degrading it to get to a nutrient source. At the same time, these models can be used to test several existing drugs' efficiency on cancer cell migration and invasion. Compared to the wound healing assays, such devices have a significant advantage through incorporation chemotactic gradients and real 3D environments. Still, it has limitations due to the lack of co-culture systems, allowing different cells' direct interaction and communication.

To improve the geometry of vascular models, a device was built which consisted of a central channel coated in Matrigel and seeded on all sides with human microvascular endothelial cells **[11]**. The transmigration of metastatic breast cancer cells MDA-MB-231 seeded on the endothelial cells was investigated. The device allowed visualization of the damage that cancer cells caused to the endothelial wall during transmigration. It was also observed that the majority of the transmigration occurred shortly after cell adhesion (within 24 h). However, different from a blood vessel, the microfluidic device had a rectangular cross-section, and this shape difference could possibly affect the interaction between cancerous and endothelial cells.

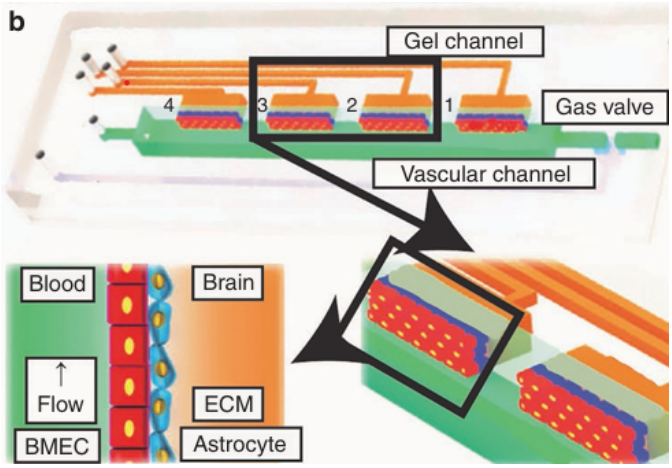

FIGURE 3 – A device designed to mimic the blood–brain barrier (BBB) and brain metastasis **[12]**.

2.3 Microfluidic metastasis-on-a-chip models for investigation of cancer extravasation

A number of organ-on-a-chip models have been developed for organs that are common secondary sites of metastases (such as lung, liver, and bone). These organ-on-a-chip models can be incorporated with vasculature extravasation models (discussed in section 'Microfluidic Investigation of Biochemical Signals in Cancer Cell Invasion'). This could allow for the creation of metastasis-on-a-chip systems that mimic the exit of cancer cells from blood vessels (extravasation) and their migration to tertiary tumor sites. Such models have provided key advantages in understanding how cancer cells interact with the surrounding microenvironment within the secondary site and in revealing mechanisms that determine the organ-specific spread of certain cancers (organ-specific metastasis).

3 A Drop in the Ocean

In this section I am going to briefly review the work done by Khan and Vanapalli in which they investigated the mechanical properties of brain cancer cells in comparison with benign cells **[6]**. Benign cells are the ones that form a clusters like cancer cells but unlike the cancer cells they form a liquid cyst around them and do not metastasize. They usually are harmless and there is no need to clear them from the body. such properties made them to be considered as the threshold between the cancerous cells and normal cells. The mechanical properties of extraneural cancer cells in comparison with benign cells have been previously studied using several techniques and the results indicate that extraneural cancer cells are softer than benign cells. Thus, this study aims to focus on brain cancer cells to achieve information about their stiffness measuring several parameters by using a microfluidic device fabricated by them. First, I am going to answer why brain cancer cells are important to investigate. Then, by addressing the material and methods of this study I will discuss their results.

3.1 Why brain cancer cells?

Primary brain tumors occur at an incidence of approximately 0.006% per capita, however, in children brain tumors account for approximately 23% of all cancers. These tumors rarely metastasize outside of the central nervous system but frequently invade nearby tissues. In some rare cases, these tumors metastasize via the cerebro-spinal fluid pathways, and blood vessels. Previous studies have been done to compare rigidity and stiffness of brain cancer cells with animal cell models using a variety of techniques including shear rheometry, scanning force microscopy, and optical stretching, however, none of these studies have examined the mechanical properties of brain tumor cells in comparison with healthy human glial cells. The importance of understanding metastasis phenomenon and preventing it by studying the Cancerous brain cells is the motivation of this study to compare these cells' properties with human benign cells.

3.2 Material and Methods

Throughout this study, they have used two methods to reach a reliable comparison between the brain cancerous cell and human benign cells. One is the **Wound Assay Healing method** and the other is using a microfluidic device named **Microfluidic Cell Squeezer (MCS)**. The Cancerous cells they used were A172 and 1321N1, and the benign cells were L0329 and L0367 glial cells.

3.2.1 Wound Assay Healing Method

In order for a cancer cell to change its position within surrounding tissues, it can use migration mechanisms that are similar to those that occur in healthy tissues during wound healing. During this method they first fabricated a monolayer of cells, then by using a P200 pipet tip they had made a scratch on this culture. The scratch was monitored over the approximately 24h for all cell lines using a camera and microscope. The wound closing velocity was determined by manually finding the average distance between the edges of each scratch at known time intervals, plotting these distances against time, and fitting to determine the slope.the comparison of this slope corresponding to different cell types would give us information about the rate of cell migration.

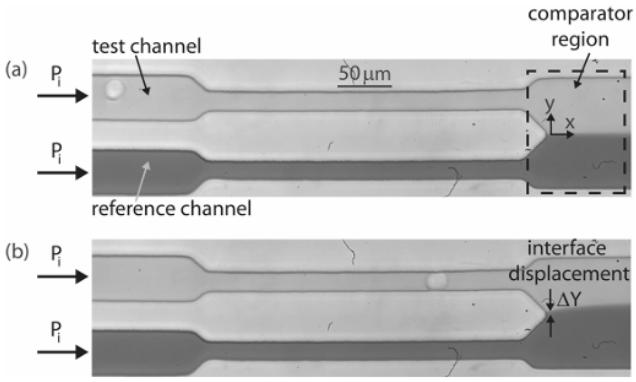

FIGURE 4 – Working principle of the microfluidic cell squeezer. (a) Balanced interface when equal driving pressures (Pi) are imposed. (b) An A172 glioblastoma cell present in the test, an interface displacement towards the channel with the higher hydrodynamic resistance or excess pressure drop.

3.2.2 MCS Device

This special device consists of two parallel microchannels : a test channel for cell analysis and a reference channel (Figure 4). Both channels inlet are connected to a flow source with same flow rate, but different fluids which are Immiscible. At the end two channels meet each other in a chamber called comparator region. If nothing is passing through the cell channel, two fluids would be at balance in comparator region. on contrary, if a cell enters the test channel there would be a pressure drop in upper channel and the interface between the two fluids moves upward toward the test channel, because of the partially obstructing cell that reduces the flow rate in the test channel. by measuring this Δy and finding the corresponding ΔP through the calibration curve, we can compare these pressure drops between varying cells and conclude about the stiffness of them.

The calibration curve has been previously been obtained by reducing the pressure in a controlled way and measuring the Δy (Figure 5). The results show that this curve is perfectly linear.

This microfluidic device were fabricated using standard soft lithography techniques. A master mould was fabricated by spin-coating a SU8-5 negative photoresist (Microchem) on a $76.2mm$ diameter silicon wafer, the thickness of which corresponds to the height of the channels ($11\mu m$). PDMS was poured into the mould and baked for $2h$ at $80°C$. The PDMS was cut and peeled from the mould, and inlet and outlet holes were punched ($0.75mm$ hole). The devices were then bonded to glass cover slides using air plasma and baked at $80°C$ for $5min$ to obtain a permanent bond. Prior to experiments, the chips were incubated with a mixture of $0.01M$ phosphate-buffered saline solution and bovine serum albumin (BSA) for $1h$ at $37°C$ to reduce the non-specific adhesion of cells to the channel walls.

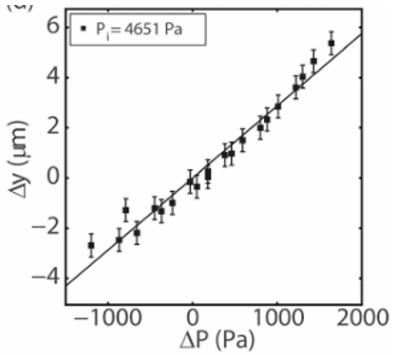

FIGURE 5 – Calibration plot of interface displacement DY for known applied excess pressure drops DP. The solid line corresponds to a linear fit. The error in these fits is $\sim 10\%$.

3.2.3 Measured Quantities

The excess pressure drop of the cells increases as the cell enters the constriction, reaches a plateau, and then decreases as the cell exits the constriction (Figure 6). They classified the excess pressure drop of the cells by the peak value obtained over the course of their passage through the constriction. The uncertainty associated with the measurement of ΔP_{peak} is taken as 10% of the peak value, as given by the calibration curve. Alongside of the Peak excess pressure drop they have also measured cell speed, elongation, and entry time into the constriction by using this device. Each of these quantities are a representative of the migration ability and of the cells.

The velocity of cells (V_c) was obtained by calculating the cells' speeds as they passed through the squeezer. To do so, they determined the centroid of the cells from the elliptical fits, and used them to calculate speed.

Entry Time (ΔT_e) the time elapsed between the point of first contact of the cells with the constriction and the point of full containment within the constriction.

The Elongation index (EI) of the cells has been determined by taking the ratio of the major axis of the cells inside the constriction to the major axis prior to entering the constriction. The major axis has been determined from elliptical fits manually performed to images of the cells during their passage through the constriction. The reported value is the maximum, typically occurring within a few cell diameters from entry into the constriction. The uncertainty associated with this measurement corresponds to a 1-pixel measurement error in manually determining the elliptical fits to the cells prior to entering and while in the constriction.

3.3 Results and Interpretation

Before continuing to results I have to introduce a quantity which corresponds to the cell's size influence in each of the measurements below. This quantity is called confinement parameter which is noted by $\frac{R_{Cell}}{R_H}$, in which the R_{Cell} is the radius of unconfined cell, and $R_H = \frac{WH}{W+H}$ is the hydraulic radius of the confined channel. Here, W and H are the constriction's width and height, respectively.

3.3.1 Cell Migration Assay

To determine the migration velocity, in Figure 6, they plotted the scratch wound displacement (rather than the wound width) against the time elapsed since the scratch was made. they found that the wound in normal human astrocyte cell lines L0329 and L0367 close more rapidly than for A172 glioblastoma or 1321N1 astrocytoma cell lines. Linear fits to the displacement vs. time data yield the following migration rates : L0367 cells migrate at a rate of $0.660.1\mu m/min$, L0329 cells migrate at a rate of $0.4260.06\mu m/min$, A172 cells migrate at a rate of $0.3960.04\mu m/min$, and 1321N1 cells migrate at a rate of $0.2660.03\mu m/min$. Uncertainties in these migration rates are determined from 95% confidence bounds on the slope of the linear fit.

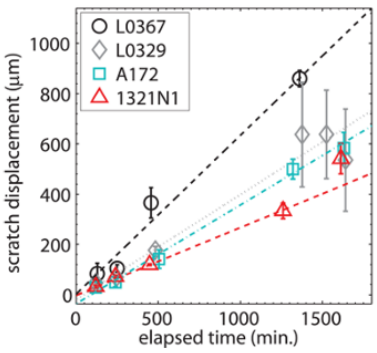

FIGURE 6 – Wound scratch displacement against time for normal healthy asctrocytes and brain cancer cells

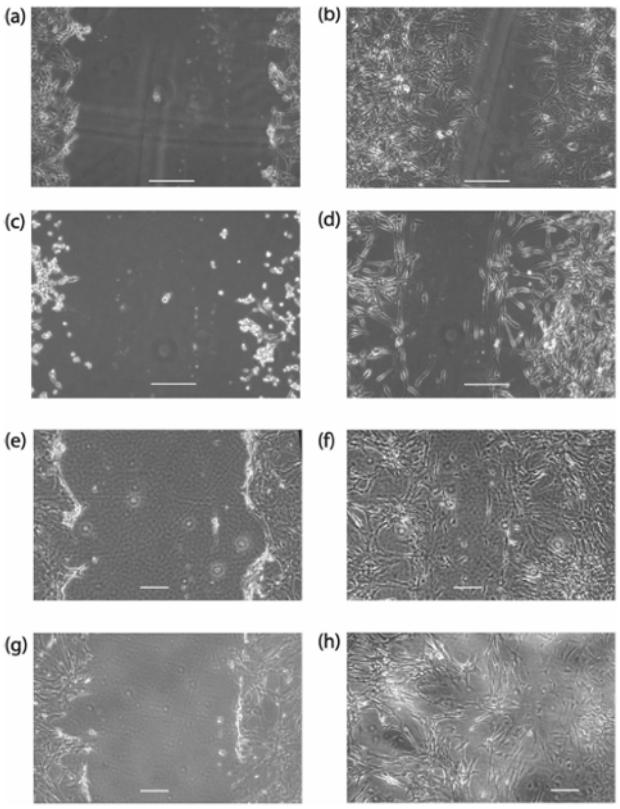

FIGURE 7 – Wound scratch assay, where A172 cells are shown in (a),(b) at the scratching time and after 1320 min, respectively. 1321N1 cells are shown in (c), (d) at the scratching time and after 1300 min, respectively. L0329 cells : (e) initial scratch and (f) 1376min later. L0367 cells : (g) initial scratch and (h) 1407min later.(The length of the scale bars is $200\mu m$.

3.3.2 Excess Peak Pressure Drop

They have observe that the ΔP increases as the cell enters the narrow passage. It reaches its highest value when the cell is about two cell diameters inside the channel and then decreases as the cell moves forward. When the cell exits the constriction, ΔP drops quickly and even becomes negative as the cell enters the comparator region, shifting the boundary between the test and reference fluids.(Figure 8) As discussed before they have used the peak value of the each cell's pressure drop. Figure 9 shows the results of this part.In order to achieve a reasonable comparison they have used two-sample Kolmogorov-Smirnov tests on all pairs of pressure drop data sets. For all cases, the null hypothesis is that the distributions for pairs of data sets are the same. For the benign cells and the A172 cancer cells, the p-value they obtained was 0.094, for benign cells and 1321N1 cancer cells it was 0.1424, and for the A172 and 1321N1 cancer cells it was 0.8608. In the case of the benign and A172 cancer cells, the p-value is low enough to reject the null hypothesis with 10% significance, however, the p-value for the benign and 1321N1 cell comparison was not low

enough to reject the null hypothesis. We also note that the p-value they obtained in comparing the two different cancer cell lines is significantly higher than comparisons between normal and cancerous cells.

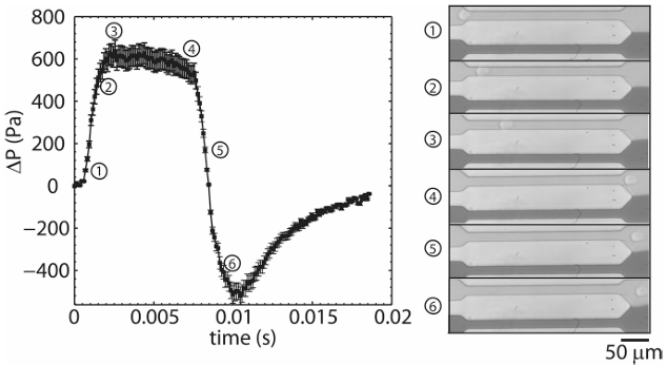

FIGURE 8 – The left panel shows how the excess pressure drop ΔP changes over time as a single A172 cell moves through the MCS. The right panel displays images of the same A172 cell at different time points, corresponding to the pressure changes shown in the left panel.

We expect that large and highly confined cells will have larger pressure drops associated with their passage through a constriction than small weakly confined cells with equal stiffness, since their increased surface area in proximity with the channel walls will result in a larger hydrodynamic resistance than for small cells. The results show that as expected for all three cell types, larger cells tend to yield higher ΔP_{Peak} values. They have observed a ten-fold increase in ΔP_{peak} as the confinement ratio is increased from 1 to 1.5. This implies that even slight confinement potentiates the mechanical resistance of glial cells. Furthermore, They also found large differences in ΔP_{peak} within cells of similar size. At a confinement ratio ~ 1.25, for example, ΔP_{peak} was determined to be $200\tilde{}2000Pa$ in cancer cell lines. This would more likely be a function of mechanical property, rather than size property, differences inherent in the cells. Results shows the statistical analysis of ΔP_{peak} values of benign and A172 cancer cells being markedly different, however benign and 1321N1 cells show no significance. This implies that, with a larger group of individuals, ΔP_{peak} could serve as a more significant discriminator for benign vs cancerous cells.

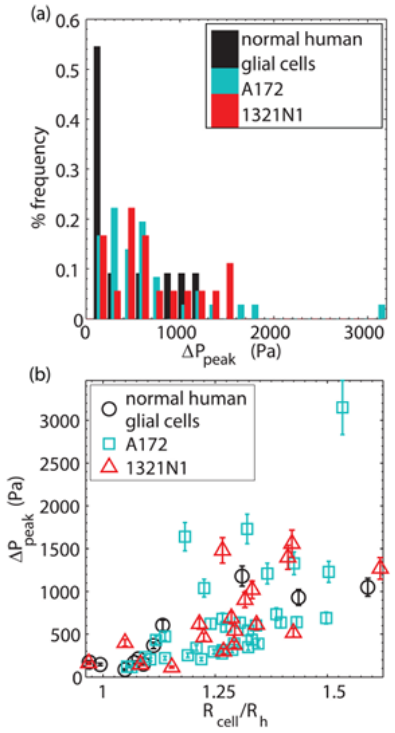

FIGURE 9 – (a) Histogram of the peak pressure drop, and (b) dependence of ΔP_{peak} on the cells' confinement parameter

3.3.3 Cell Speed in the Squeezer

Additionally, They also evaluated the speed of the cells as they traversed the narrow constriction $(4.5\mu m)$ and plotted the results in the form of histograms shown in Figure 10(a). According to our statistical test (Kolmogorov-Smirnov test), the speeds of A172 and 1321N1 cancer cells were significantly different (significance level = 10%). However, when comparing cancer cells versus benign, there was no difference, meaning that their speed distributions were statistically similar. Next, we tested if cell speed was altered by confinement (Figure 10(b)). While ΔP_{peak} increased with confinement, cell speed was scatter without a clear confinement pattern (Supplementary Fig. 2). The viscous, deformable object speed in microchannels depends on two factors :

1. The fluid around the cell pushes the cell forward.
2. The interaction of the cell to the walls of the channel.

This is because our results showed the fluid moved much more slowly than the cells (20%–80% slower). But cancerous and benign cells or different levels of confinement produced largely the same speed while neighborly. This indicates that the frictional forces between the cells and channel walls were comparable across different cell types.

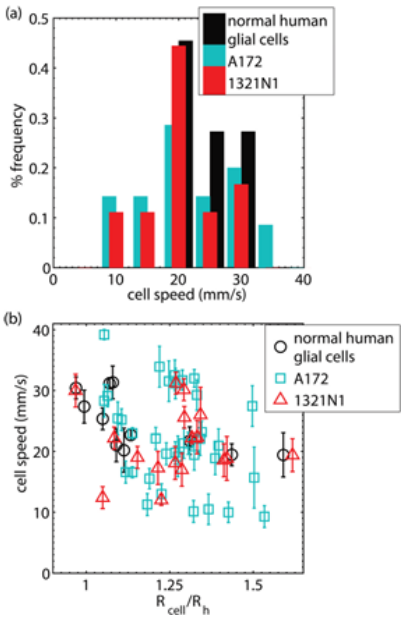

FIGURE 10 – (a) Histograms of the cell speed associated with the passage of a cell through a narrow microfluidic constriction, and (b) the dependence of cell speed on the cell's confinement.

3.3.4 Cell Entry Time into the Microchannel

In their research inbound time for cancerous and non-cancerous glial cells was obtained as depicted in Figure 11. As with other studies, bigger cells entered more slowly. Astrocytes are normal human glial cell types, and we can see that these cell types are more likely to traverse through the constriction than the cancerous glial cell types. The difference is not significant on both the cancerous cell lines (A172 and 1321N1). To our surprise, this result is the opposite of what has been seen in breast cancer studies, where benign cells incorporated more slowly than malignant ones. These findings indicate that normal human astrocytes are more time-dependent and softer than cancerous glioblastoma and astrocytoma cells, and highlight differences in the mechanical properties of brain tumors as compared to other cancer types.

3.3.5 Cell Elongation

They found that in this part null-hypothesis cannot be rejected, indicating that both cancerous and benign cells approach similar shape deformations under the same driving pressure. Finally, we analyzed the EI variation with confinement for all cell types (Figure 12(b)). The data indicate that EI increases as confinement increases, such that smaller cells are stretched less than larger cells in the constriction. As expected from the results in Figure 12(a), we can conclude from Figure 12(b) that no significant difference exists in the elongation of cancerous vs normal glial cells.

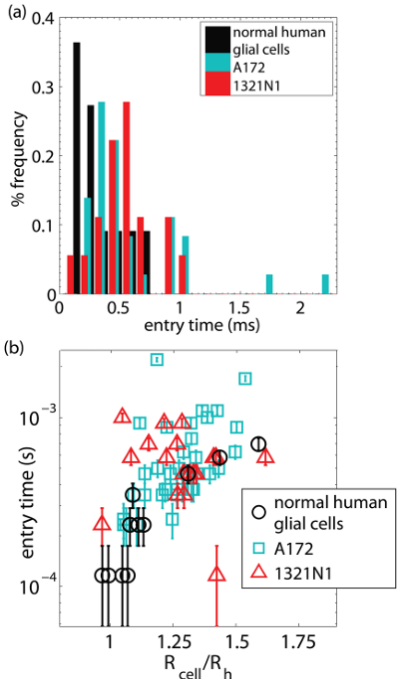

FIGURE 11 – (a) Histograms of the cells' entry times into the narrow constriction, and (b) the dependence of entry time on the cell's confinement

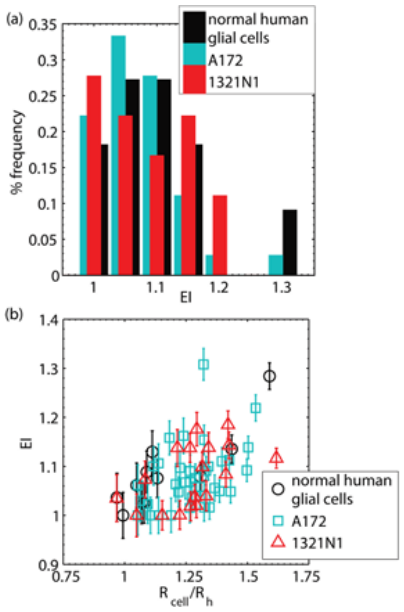

FIGURE 12 – (a) Histogram of the EI, and (b) dependence of EI on the cells' confinement.

Figure 13 shows the summary of the results in which we can see the rejection or not-rejection of null-hypothesis for each of cell line's comparison for different measured parameters.

Measured quantity	Cell line 1	Cell line 2	Null hypothesis rejected (10% significance)	Statistical significance (p-value)
ΔP_{peak}	Benign	A172	Yes	0.0904
ΔP_{peak}	Benign	1321N1	No	0.1424
ΔP_{peak}	1321N1	A172	No	0.8608
EI	Benign	A172	No	0.9999
EI	Benign	1321N1	No	0.9770
EI	1321N1	A172	No	0.5384
Speed	Benign	A172	No	0.2490
Speed	Benign	1321N1	No	0.9770
Speed	1321N1	A172	Yes	0.0425
Entry time	Benign	A172	Yes	0.0065
Entry time	Benign	1321N1	Yes	0.0669
Entry time	1321N1	A172	No	0.7061

FIGURE 13 – Statistical significance of differences between cell lines for measured distributions of excess pressure drop ΔP_{peak}, EI, cell speed in constriction, and entry times into the constriction.

4 Conclusion

Microfluidic technology for cancer metastasis study allows to analyze the physical and chemical conditions under which tumor metastasis occurs. By replicating the in vivo microenvironment with high precision, microfluidic devices enable researchers to analyze key aspects of metastasis, such as cell migration, extravasation, and interactions with the surrounding tissue. The ability to test drug responses in a controlled 3D environment also provides a more reliable platform for therapeutic screening compared to traditional in vitro assays.

One key takeaway from this review is that not all cancers behave the same way. While many cancers become more aggressive by becoming softer and more flexible, brain cancer cells tend to be stiffer, which changes how they migrate, possibly due to the unique CNS environment. We approved this from various approaches and measurements of quantities which somehow represents the stiffness and ability of migration of these cells and by comparing those with benign cells. Also, The statistical method used for comparison showed that we were able to compare cell lines better by the **Entry Time** measurement. In general, the findings of this study, highlights the importance of tailoring cancer research and treatments to specific tumor types rather than applying a one-size-fits-all approach. As research advances, the integration of organ-on-a-chip models and co-culture systems will provide an even clearer picture of how metastasis works, helping us move closer to more effective, personalized cancer therapies.

Also, during this review we were introduced to a new microfluidic device named The Microfluidic Cell Squeezer (MCS), which was an effective tool for studying mechanics of cells. So, Microfluidic devices can be engineered with multiple squeezing channels to enable high throughput mechanical characterization of tumor cells. Future studies should be directed towards probing the detailed micromechanics of tumor cells, including the role of the nucleus.

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