Tumor Cell Deformability in the Metastatic Cascade

by

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Abstract

During the process of metastasis, tumor cells must undergo changes that enable them to detach from a tumor, exit surrounding tissue during intravasation, enter into circulation and eventually stop at a distant site for extravasation. Here, we measure the physical changes in the deformability of tumor cells, indicated by the length of time required to pass through a microfluidic constriction in a suspended microchannel resonator (SMR), as related to different stages of the metastatic process—particularly, in an epithelial-mesenchymal transition (EMT) and existence in the circulation.

We find that a mesenchymal population of murine tumor cells (MMTV-PyMT) that had undergone a spontaneous EMT at the primary tumor site were more deformable than the parental population of epithelial cells. In contrast, MMTV-PyMT and Ep5 murine breast carcinoma cells that had received signaling from platelets to undergo an epithelial-mesenchymal-like transition maintained the same deformability or became less deformable, respectively. In all cases, however, epithelial and mesenchymal tumor cells both take much longer to pass through a constriction than typical blood cells, as confirmed by examining various human cancer cell lines (H1975, SKBR-3, MDA-MB231, PC3-9). Using a syngeneic mouse tumor model, we find that cells that are able to exit a tumor and enter the circulation are not required to be particularly more deformable than the cells initially injected into the mouse. However, in a limited study of prostate cancer patients, various circulating tumor cells (CTCs) can pass through a constriction quickly because some are relatively small in size, while others are more deformable than typical tumor cell lines and more mechanically similar to blood cells.

Nonetheless, due to the ambiguity in cell identity when a heterogeneous sample like blood is assessed by the SMR, there was a need to correlate each cell’s precision biophysical measurement to its molecular expression. I thus developed a technique whereby cells can be sorted off-chip based on their passage time and/or buoyant mass characteristics, and collected into a 96-well plate. The proof-of-principle is demonstrated by sorting and collecting cells from cell line-spiked blood samples as well as a metastatic prostate cancer patient blood sample, classifying them by their surface protein expression and relating them to distinct SMR signal trajectories. Taken together, our results provide impetus for further studies on the mechanical properties of CTCs as well as the future utilization of this platform for other types of biophysical-molecular characterizations.

Thesis Supervisor: Scott R. Manalis
Title: Professor of Biological and Mechanical Engineering
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Chapter 1

Introduction

1.1 Cancer metastasis

Metastasis involves a cascade of events, where carcinoma cells undergo molecular and cellular changes as they journey away from the primary tumor and travel to a distant site to colonize a new tumor [1–3]. During this journey, cells that were once embedded within a tumor detach and undergo potential elastic deformation to penetrate surrounding tissue, including endothelial cell junctions, to intravasate into the lymphatic or circulatory system (Fig. 1-1). As tumor cells circulate in the bloodstream as circulating tumor cells (CTCs), they squeeze through small capillaries and endure harsh flow conditions, leaving only a fraction of them to survive and adhere at a distant site for subsequent extravasation [4]. Then, among those that are able to extravasate into surrounding tissue, depending on the conditions of the microenvironment as well as traits acquired by the carcinoma cells themselves, some advance to seed and colonize new metastatic lesions [4,5].

![Fig. 1-1. Simplified schematic of the metastatic cascade](image)

Cells within a primary tumor undergo changes such as an epithelial-mesenchymal transition (EMT), detach from the tumor, intravasate into circulation, where they become circulating tumor cells (CTCs). Then, some CTCs are able to extravasate, potentially undergo a mesenchymal-epithelial transition (MET), and either reseed the original tumor or seed a new tumor at a metastatic site.

1.1.1 Epithelial-mesenchymal transition

During the metastatic process, it is thought that tumor cells undergo cell-biological changes, such as progression through an epithelial-mesenchymal transition (EMT). In the course of this program, epithelial cells tend to become less differentiated, reducing intercellular adhesion, and acquire
mesenchymal traits, such as increased motility and invasiveness (Fig. 1-2) [6–10]. The phenotypic change frequently involves the loss of E-cadherin, an adhesion molecule, and often includes an increase in mesenchymal markers, such as N-cadherin and vimentin [7,8]. Such transitions underlie epithelial cell plasticity and enable several developmental processes in vivo.

![Diagram of Epithelial-mesenchymal transition](image)

**Fig. 1-2.** Epithelial-mesenchymal transition
An EMT involves cell-biological changes, where an epithelial cell undergoes a progressive loss of epithelial markers and gain of mesenchymal markers, rendering the cell less differentiated and more invasive. This figure is adapted from Ref. [7].

EMT has already been known to play key roles during embryonic development, including embryo implantation as well as gastrulation [11]. It has also been shown to occur during tissue repair and inflammation, where inflammatory stress induces epithelial cells to advance to various degrees of EMT, or “partial EMTs” [7]. More recently, however, EMT has gained increasing attention due to its association to human diseases, including the onset and progression of carcinomas [6–8]. For example, it has been shown in a pancreatic ductal adenocarcinoma mouse model that EMT precedes frank tumorigenesis [12]. In humans, mesenchymal markers have been identified in carcinoma biopsies [13]. Additionally, a recent study has discovered CTCs in breast cancer patients that exhibited a range of epithelial to mesenchymal expression; indeed, in one case study, the breast cancer patients exhibited a higher fraction of CTCs with mesenchymal markers when the disease was actively progressing [14]. Furthermore, EMT has been linked to cancer stem cells and increased therapeutic resistance [6,15].

The phenotypic transition can be induced by environmental cues such as growth factors, cytokines, certain physiological conditions, and other interactions in the tumor microenvironment. One of the most well characterized inducers of EMT is the transforming growth factor-β (TGFβ) family of cytokines. TGFβ is known to effect several pathways, including phosphorylation of SMAD transcription factors, which can regulate cell polarity, tight junction formation, and can associate with Zeb proteins to repress E-cadherin expression [6,15]. Notch signaling can also induce EMT through the nuclear factor-κB (NFκB) pathway. NFκB regulates production of
vascular endothelial growth factor (VEGF), which aids in angiogenesis, and matrix metalloproteinases (MMPs), which can degrade basement membrane and extracellular matrix components to aid in tumor cell invasion and metastasis [16–18]. In addition to various signaling mechanisms, the physiological condition of hypoxia can also induce EMT in tumors via various signaling mechanisms (HIF1α, SNAIl, TWIST1, NFκB) [19]. Moreover, the tumor microenvironment, as dictated by tumor-stromal cell interactions can also trigger EMT in carcinoma cells. Indeed, multiple EMT markers have been found at the tumor-stroma interface [13,20]. Last, the direct contact between tumor cells and platelets induces an epithelial-mesenchymal-like (EMT-like) transition, involving signaling along both the TGFβ and NFκB pathways [21–25]. Thus, various triggers may work together in increasing tumor cell invasiveness and malignancy.

1.1.2 Circulating tumor cells

Carcinoma cells that have escaped into circulation, known as CTCs, have drawn increasing interest in recent years due to their potential in cancer prognosis as well as the information they hold regarding a patient’s tumors [26,27]. As some tumors are difficult or unfeasible to biopsy, it would be beneficial if CTCs from a simple blood draw could give doctors a window into the progression of a patient’s tumors as well as their genetic mutations over time. Such information would aid in determining more appropriate and personalized therapies. In spite of rapidly advancing research, still much is unknown about CTCs, and although there are some standards in place, an optimized and adequate set of properties by which to identify them has not yet been fully determined. Even with current methods, such as the FDA (Food and Drug Administration) approved CellSearch system (Veridex), which selects cells from blood based on the surface expression of epithelial cell adhesion molecule (EpCAM), CTCs are very rare in the blood (5 CTCs are considered significant in 7.5mL blood) and not trivial to isolate. In fact, in one mL of blood there are billions of red blood cells and millions of white blood cells, yet only a few, tens, or at most thousands, of CTCs. Thus, scientists and engineers have been developing a variety of methods to isolate CTCs based on a number of cellular and molecular properties.

The most commonly used methods for CTC isolation are based upon antibody detection of cell surface antigens. Since epithelial cells express EpCAM, whereas blood cells do not, EpCAM is used to enrich CTCs from blood samples. One platform utilizing this strategy is the previously mentioned CellSearch system, which employs ferrofluid nanoparticles coated with anti-EpCAM antibodies to capture the cells, and then stains them for cytokeratin and CD45 to distinguish between epithelial cells and leukocytes [28,29]. Other examples include microfluidic devices that are coated with anti-EpCAM antibody, where captured CTCs either remain adhered or are released from the chip, and can be stained for other epithelial markers for further imaging analysis [30–32]. Although the number of cells captured based on EpCAM expression have been shown to possess prognostic value for some cancers, it is not known what role these EpCAM expressing cells have
in metastasis and whether another non-EpCAM expressing population of CTCs may provide additional information [33,34].

In fact, recent reports have shown that some breast cancer cell lines do not express EpCAM and are thus not captured by EpCAM-based isolation techniques [35–37]. Furthermore, in a study involving an autochthonous pancreatic ductal adenocarcinoma mouse model, CTCs were found to maintain a mesenchymal phenotype and bear stem cell properties [12]. As mentioned previously, a case study in breast cancer has also shown CTCs bearing mesenchymal characteristics [14]. In order to avoid biases in positively selecting for surface markers, other methods have been employed, such as nucleic-acid base detection. An increased level of DNA in the plasma, as measured by quantitative PCR, has been shown to correlate to cancer patients over healthy individuals, and may correspond to the presence of CTCs [38–40]. However, the origin of such DNA is unclear and may produce false positive findings due to the shedding of dying cells directly from tumors, exosomes, and non-malignant epithelial cells released from an invasive procedure [28,29]. In addition, physical methods have been used to isolate CTCs, such as separating cells by size. Two examples include a filtration system known as Isolation by Size of Epithelial Tumor cells (ISET, Rarecells) [41], and dean flow fractionation, which involves a spiral channel employing centrifugal forces [42]. However, distinguishing between sizes does not provide sufficient specificity toward the cells being retained since small CTCs (similar in size to most leukocytes) may be lost, while large leukocytes may be enriched for [29,31]. Last, negative depletion is a method by which white blood cells are removed by anti-CD45 antibodies, thereby enriching the blood sample for CTCs [43]. One platform is the CTC-iChip, which removes red blood cells by size-dependent deterministic lateral displacement and removes white blood cells by labeling them with magnetic beads, targeting CD45 and CD15 [44,45]. Although in general, negative depletion methods may leave impurities in the enriched CTC samples, they may prove to be one of the least biased methods for isolating CTCs [28,46].

Currently, only the enumeration of CTCs (largely based on EpCAM expression) has been recognized to have prognostic value in particular carcinomas. Current efforts have mostly been focusing on molecular analyses of CTCs, including genetic and protein expression, as they begin to uncover their heterogeneity and potential function in metastasis. Genetic alterations, such as in chromosomal translocations, transcriptional profile variations, and mutations in the exome have been demonstrated, and may provide insight into the diversity of tumor cells as well as mutations occurring at the tumor sites [47–50]. Analysis of protein expression, often evaluated by immunofluorescence or immunohistochemistry, has included assessment of a proliferative index by Ki-67, which increase has been correlated to resistance to certain prostate cancer therapies [51]. Another protein of interest is HER2, which may be an important indicator of HER2 status changes during breast cancer progression [50]. Also, androgen receptor (AR) signaling in prostate cancer, based on expression levels of prostate-specific antigen (PSA) and prostate-specific membrane antigen (PSMA), has been shown to relate to patient survival [52]. Although much effort is underway to study the molecular characteristics of CTCs and their implications, not much has been studied from a holistic biophysical point of view. While specific genes and proteins may be
involved in particular cancers or their subtypes, it is possible that the biophysical properties of cells, such as their deformability, may be more generally applicable across multiple cancer types. Although mechanical differences between metastatic and nonmetastatic cell lines have been demonstrated, only few precision measurements have been made on patient CTCs, with little implication on what properties may be necessary for them to adapt to the circulation [53–56].

1.2 Single-cell deformability

For decades, scientists have been intrigued by the physical characteristics of living cells, including their stiffness or deformability [57]. Using various methods, they established that cell deformability can identify changes in cell phenotype, including those in differentiation and malignancy [55,58–60]. Mechanical changes have even been discovered to provide diagnostic value in oral cancer [56]. From these studies, it has been found that often, more malignant cells tend to be more deformable than nonmalignant cells.

1.2.1 Existing methods for measuring single-cell deformability

There are now copious methods by which one can measure the deformability of single cells [61]. Some techniques probe a portion of a cell or its surface, such as atomic force microscopy (AFM) and microrheological methods. AFM consists of a sharp tip at the end of a cantilever, which can apply small forces (pN to μN) to locally deform a cell’s surface. The depth of indentation relative to the applied force provides information regarding the local elasticity (Young’s modulus) of the cell [62–64]. Meanwhile, microrheology involves the tracking of particles or beads that are either adhered to a cell’s surface or introduced within the cell. Based on their motion due to an applied magnetic field or to Brownian motion, viscoelastic properties of local regions of a cell can be determined [65–69].

Other techniques for measuring single-cell deformability, including optical stretching, hydrodynamic deformation, micropipette aspiration, and microfluidic constriction devices, apply a stress to the entire cell holistically. To accurately measure cell deformability, each of these methods must also be able to account for cell size. The optical stretcher consists of two laser beams, which apply a stretching surface force to a cell that is placed between them. The change in shape experienced by the cell over a given time is recorded as the compliance of the cell, having been shown to be indicative of cytoskeletal strength [70,71]. Then, rather than using laser beams, hydrodynamic stretching applies a stretching force to the cell with fluid flow. This high throughput method (2,000 cells/s) captures images of the deformation of each cell, and based on the cell shape, obtains a metric for cell deformability and size [58,72]. Hydrodynamic shear stress has also been used to assess cell mechanics in a high throughput fashion (100 cells/s) [73]. Next, micropipette aspiration involves suctioning a cell either partially or fully into a micropipette. Observation of changes in a cell’s geometry provides insight into its elastic and viscoelastic properties [57,74]. Finally, there are also microfluidic constriction devices, which, similar to micropipette aspiration,
consist of a small orifice through which cells must pass by deformation. The throughput of microfluidic devices, however, tends to be higher than that of micropipette aspiration due to its automated cell loading capabilities. The amount of time the cell takes to pass through the constriction can be considered as a metric for cell deformability, and has been shown to relate to cytoskeletal structure [59,75–77]. One such microfluidic device is the suspended microchannel resonator (SMR), which has the ability to determine the size of each cell as well as parse out the relative contributions of deformability and friction to the passage of a cell through the constricted channel, as described in the following section [59].

1.2.2 The suspended microchannel resonator (SMR) with constriction*

The SMR consists of a hollow microchannel embedded in a silicon cantilever, whose resonant frequency is detected by the deflection of a laser beam [78]. [We have integrated a constriction near the apex of a SMR as shown in Fig. 1-3A.] In each experiment, a solution with cells that are denser than the surrounding fluid is flowed into the device. Once a cell enters the SMR, the resonant frequency is lowered by an amount that depends on its buoyant mass and position away from the resonator base (Fig. 1-3B) [78]. By tracking the resonant frequency as a cell traverses through the SMR, the position of its center of mass along the channel and its buoyant mass can be measured with a precision near 100 nm and 1 pg, respectively, for a cell that weighs ~100 pg and travels through the SMR in ~1 s. As a result, a cell’s buoyant mass, passage time, velocity upon entering the constriction (entry velocity), and velocity as it transits through the constriction (transit velocity) can be extracted with a throughput of a few thousand cells per hour. Buoyant mass, which is the metric we use in this study for cell size, is defined by the product of the cell’s volume and its density difference from the surrounding fluid.

Using the SMR with the integrated constriction, we demonstrate that precise, single-cell buoyant mass measurements in conjunction with passage time information enable the distinction between cell lines bearing different physical characteristics. More specifically, these combined measurements reveal differences between cell lines arising from blood and epithelial tissue, as well as between cell lines having varying metastatic potential. To assess factors affecting cell passage through the constriction, we further show that entry and transit velocity measurements enable us to identify the relative importance of deformability and surface friction, respectively. Changing the deformability of the cell by perturbing its cytoskeleton primarily alters the entry velocity, whereas changing the surface friction by immobilizing positive charges on the constriction’s walls primarily alters the transit velocity. To demonstrate the insight that these parameters provide, we compare the properties of both mouse and human cancer cell lines having

* This section as well as its subsections are individual excerpts taken directly from a manuscript, the writing to which I contributed heavily: Byun, S., Son, S., Amodei, D., Cermak, N., Shaw, J. et al. “Characterizing deformability and surface friction of cancer cells.” PNAS. 2013. 110(19):7580-5. Author contributions are listed in the publication. Changes to the published text include reference numbers, figure numbers, and text included in brackets.
known metastatic potentials. When accounting for cell buoyant mass, we find that cells possessing higher metastatic potential exhibit faster entry velocities than cells with lower metastatic potential. However, in some cases, the increase in transit velocities associated with faster entry velocities was considerably greater than expected, suggesting that reduced friction may be a factor in enabling invasive cancer cells to efficiently squeeze through tight spaces.

Fig. 1-3. Schematic diagram of the instrument and data extracted from the measurement
A) Suspended microchannel resonator (SMR) with a constriction (6 μm wide, 15 μm deep, and 50 μm long) located at the apex. A cell passing through an embedded microfluidic channel is deformed as it flows into the constriction. Numbers 1–5 indicate different positions within the microchannel to demonstrate the trajectory of a cell flowing inside the channel. B) The resonant frequency response of the SMR as the cell passes through the microfluidic channel. The numbers 1–5 correspond to the position of the cell in the cantilever, as marked in A. The height of the peak corresponds to the buoyant mass of the cell (1→2). The cell slows down as it deforms to enter the constriction (entry), and then speeds up as it travels through the constriction (transit). The passage time corresponds to the sum of the entry and transit times (3→4). C) Power law dependence of passage time versus buoyant mass for the H1975 cell line (n = 967). Measurements were acquired with a PEG-coated channel surface and using a pressure drop of 1.8 psi.

1.2.2.1 Single-cell buoyant mass and passage time through a constriction
We first measured the buoyant mass and passage times of hundreds of single cells from a human lung adenocarcinoma cell line, H1975 (Fig. 1-3C), to validate our method. As expected, a cell’s passage time through the constriction has a power law relationship to its buoyant mass [79].
Interestingly, the range of passage times spans nearly four orders of magnitude as it changes with cell buoyant mass. In addition, the passage time of cells having the same buoyant mass varies by up to an order of magnitude, suggesting the influence of other effects. Compared with other approaches for measuring cell size and passage time through a constriction [75,77,80,81], our results show a thousand-fold larger range in passage time across the population and a nearly 10-fold larger variation in passage time for cells of similar buoyant mass. One possible explanation for this discrepancy is that, because the passage times in these previous studies were typically on the millisecond scale and below [77,80], the relatively higher strain rates diminished the range in the mechanical properties. Although it seems surprising that cells of similar buoyant mass could have such a significant variation in passage time, time lapse microscopy of a cell passing through multiple constrictions in existing literature [82] suggests that, in addition to inherent biological variation, the orientation of the cell upon entry into the constriction could give rise to this spread.

1.2.2.2 Entry and transit velocities revealing relative contributions of deformability and surface friction

To assess the components that govern passage time, the entry and transit velocities were extracted from the SMR measurements. The passage of each cell through the constricted microchannel comprises the initial deformation of the cell to enter the constriction as well as the subsequent transit of the cell through the constriction. The velocity of each cell during its entry and transit can be obtained by monitoring the position of its center of mass within the microchannel (Fig. 1-4 A and B), as given by the resonant frequency shift of the cantilever [83]. Both entry and transit velocities have a power law dependence on the buoyant mass, similar to the passage time, but these velocities decrease with increasing buoyant mass (Fig. 1-4C). Also, for a given cell, the entry velocity is slower than the transit velocity. However, the difference between the entry and transit velocities is less pronounced for smaller cells, because smaller cells require less time to deform into the constriction in comparison with larger cells. Hence, in complement with passage time information, the entry and transit velocities can provide more insight into cellular properties governing the passage through the constriction.

Next, the effects of deformability and surface friction on the passage of cells through the constriction were assessed by measuring H1975 cells under two separate conditions—having perturbed its cytoskeleton with latrunculin B (LatB), and having coated the microchannel surface with positively charged poly-L-lysine (PLL) [Fig. 1-5, 1-6]. First, as expected, the treatment with LatB decreased the passage time of the cells (Fig. 1-5), corresponding to the LatB-induced increase in cell deformability by the disruption of actin filaments [84]. Furthermore, the LatB treatment increased both entry and transit velocities, with the relative increase in entry velocity being greater than that of transit velocity (Fig. 1-6 A–C). Although the small change in transit velocity indicates that a change in cell stiffness can influence the friction because the normal force of the cell against the channel wall is altered [85], the greater change in entry velocity is consistent with the fact that perturbing the viscoelastic properties of the cell affects the passage most when the deformation of
the cell is critical, i.e., at the entry rather than during transit. A similar behavior was observed when MEF cells were treated with nocodazole (Fig. A-1), further demonstrating that a cytoskeletal deformability change in the cell corresponds to a larger shift in entry velocity than transit velocity.

Fig. 1-4. Extracting entry and transit velocities from single cell measurement
A) The resonant frequency response (positions 3–5 in Fig. 1-3B) is converted to the normalized position of the cell in the cantilever and plotted versus time. The length of the cantilever was normalized to 1 to represent the cell’s position, where 1 and 0 correspond to the tip and base, respectively. B) Cell velocity is obtained by taking the time derivative of the normalized position. Entry and transit velocities are extracted at specific locations that correspond to the entrance and the inside of the constriction, respectively. C) Entry (green) and transit (orange) velocities versus buoyant mass for the data set from Fig. 1-3C.

Coating the microchannel constriction with PLL increased the passage time of the cells (Fig. 1-5B), corresponding to the increased interaction of the negatively charged cell surface with the positively charged PLL-coated channel surface than with the neutral PLL-graft-PEG-coated surface. The PLL coating decreased both the entry and transit velocities, but with a larger change in transit velocity (Fig. 1-6 D–F). Although the surface interaction during the deformation at the constriction entry was nontrivial, the effect was greatest when the contact area between the cell and the channel wall was maximum, i.e., during the cell’s transit through the constriction. The transit velocity thus represents a friction measurement that encapsulates effects contributing to the normal force exerted by the cell on the channel wall, as well as interactions between the surface of the cell and the channel. The experiment involving a PLL surface coating demonstrates that enhancing surface interactions, such as those governed by charge, alters the transit velocity more than entry velocity. Similar results were obtained for various other cell lines, such as HCC827, T\text{Met}, and T\text{nonMet} (Fig. A-2). Hence, even though deformability and friction are not completely orthogonal in our measurements, the relative differences in entry and transit velocities provide a metric for the relative importance of deformability and friction during the passage of a cell through a constriction.
Fig. 1-5. Changes in the passage time of H1975 cells after perturbing either deformability or microchannel surface charges
A) Passage time versus buoyant mass for H1975 untreated (blue) and treated with latrunculin B (LatB) (red). Treatment with LatB decreases the passage time. B) Passage time versus buoyant mass for H1975 for a microchannel surface coated with positively charged poly-L-lysine (PLL) (blue) and neutral PEG (red). Coating with PLL increases the passage time. The data in this figure match with those of Fig. 1-6.
Fig. 1-6. Changes in the entry and transit velocities of H1975 cells after perturbing either deformability or microchannel surface charge

A) Entry velocity and B) transit velocity versus buoyant mass for H1975 untreated (blue, n = 843) and treated with LatB (red, n = 907, 5 μg/mL for 30 min) measured in a PEG-coated channel. Treatment with LatB decreases the passage time of H1975 and induces a larger shift in entry velocity than transit velocity. C) A ratio of velocities from the two conditions was calculated as in Ref [59]. Changing the deformability of the cell by perturbing its actin cytoskeleton induces a 3.8-fold increase in the entry velocity, and only a 1.5-fold increase in the transit velocity. D) Entry velocity and E) transit velocity versus buoyant mass for H1975 cells passing through a microchannel whose surface is coated with positively charged PLL (blue, n = 345) or neutral PEG (red, n = 649). PLL increases the passage time and results in a greater shift in transit velocity than entry velocity. F) Changing the surface friction from PEG to PLL caused entry velocity to decrease 2.3-fold, and transit velocity to decrease 4.7-fold. Error bars represent 95% confidence intervals. All measurements were acquired using a pressure drop of 1.8 psi. [For an explanation of calculating ratios of velocities, see Fig. 2-3 and Ref [59].]
1.2.2.3 Cancer cells reveal differences in relative changes in entry and transit velocities

We also found that the relative importance of the cell’s deformability and surface friction to its passage through the constriction could be a unique indicator of cell state. The deformation and friction properties of cancer cells having different known metastatic potentials were characterized by entry and transit velocities on a PEG-coated channel (Fig. 1-7). In addition to having shorter passage times [59], all three cell lines with higher metastatic potential exhibit faster entry and transit velocities. Interestingly, the relative differences in entry and transit velocities from each pair showed distinct patterns. Expressing the Nkx2-1 transcription factor in T\textsubscript{Met} led to a change primarily in entry velocity, which is similar to what we observed in altering the cytoskeletal deformability of the cells with LatB (Fig. 1-6C). In contrast, T\textsubscript{Met} versus T\textsubscript{nonMet} showed significant differences in both entry and transit velocities, suggesting that changes in friction account for more of the difference between this pair of cells than between the pair with a single genetic alteration (T\textsubscript{Met} versus T\textsubscript{Met-Nkx2-1}). H1975 versus HCC827 also demonstrated that a considerable change in the transit velocity is associated with the entry velocity. These examples suggest that reduced friction may play a role in enabling invasive cancer cells to effectively squeeze through tight spaces. It is possible that such changes in friction may be related to differences in factors contributing to the normal force of the cell on the channel wall, such as cell relaxation times. Additionally, the observed changes may be caused by glycocalyx characteristics, such as the expression of particular cell surface molecules like sialic acid, because it is already known that increased expression of sialic acid may be accompanied with a higher metastatic potential in cancer cells [86].
**Fig. 1-7.** Entry and transit velocities of cancer cell lines having different metastatic potentials

Three pairs of cancer cell lines having different metastatic potentials (T<sub>Met</sub> versus T<sub>Met-Nkx2-1</sub>, red; T<sub>Met</sub> versus T<sub>nonMet</sub>, green; and H1975 versus HCC827, blue) were compared by measuring the changes in entry velocity (V<sub>E</sub>) and transit velocity (V<sub>T</sub>) with a PEG-coated channel surface. A) Ratio of V<sub>E</sub> and ratio of V<sub>T</sub> for three pairs of cancer cell lines. V<sub>E</sub> and V<sub>T</sub> ratios connected by a line represent one replicate. In contrast to T<sub>Met</sub> versus T<sub>Met-Nkx2-1</sub>, T<sub>Met</sub> versus T<sub>nonMet</sub>, and H1975 versus HCC827 show that a significant change in transit velocity is associated with a change in entry velocity, suggesting that the role of friction is more significant in those pairs. T<sub>Met</sub> versus T<sub>Met-Nkx2-1</sub>, T<sub>Met</sub> versus T<sub>nonMet</sub>, and H1975 versus HCC827 were repeated from different cultures three, six, and three times, respectively. Error bars represent 95% confidence intervals. B) For each measurement, the ratio of V<sub>E</sub> divided by the ratio of V<sub>T</sub> is shown, which confirms that the proportional change in V<sub>E</sub> relative to V<sub>T</sub> was significantly different among the three pairs (*p < 0.05, Mann-Whitney-Wilcoxon test). Measurements were acquired using a pressure drop of 0.9 psi for the mouse cell lines (T<sub>Met</sub>, T<sub>Met-Nkx2-1</sub>, T<sub>nonMet</sub>) and a higher drop of 1.8 psi for the human cell lines (H1975, HCC827) to account for their larger size.

### 1.3 Measuring tumor cell deformability with the SMR

As we have shown, the SMR can precisely weigh single cells and record their position along the channel. This enables us to decouple a cell’s size from its passage time through a constriction, as a measure of its deformability. Furthermore, the velocities of the cell, both as it enters the constriction and as it transits through after the initial deformation, can provide additional information regarding the relative significance between deformability and surface friction. The aforementioned demonstration of the technique involves comparing cell lines of varying metastatic potential. In the following chapters, we utilize the SMR to further unveil deformability characteristics specifically when carcinoma cells undergo EMT as well as when they are in circulation, comparing their properties to those of other cells naturally existing in circulation. These precise mechanical measurements of tumor cells aim to provide a physical perspective and understanding to cancer cell mechanics in various stages of the metastatic cascade.
Chapter 2

Tumor Cell Deformability Changes in an Epithelial-Mesenchymal Transition

2.1 Introduction

Over the past decade, the importance and role of the EMT program in the onset and progression of carcinomas have become increasingly clear [6–8]. Mesenchymal markers have been identified both in carcinoma biopsies as well as in circulating tumor cells during disease progression [13,14]. Furthermore, it has been found that direct contact with platelets induces an epithelial-mesenchymal-like (EMT-like) transition in tumor cells, rendering them more highly metastatic [21–25]. Thus, it is of interest to determine physical changes that occur concomitantly with these cell-biological changes that enable carcinoma cells to enhance their malignancy both in the primary tumor site as well as in circulation [87,88]. Previous studies of the mechanical properties of cells have employed various methods to probe cancer cells from cell lines or body fluids, demonstrating that highly metastatic cells are often more deformable than weakly metastatic cells [55,58,59,72,89–95]. Here, we utilize an SMR with a 6μm-wide constriction to measure active physical changes displayed by carcinoma cells after they become more malignant, focusing specifically on how EMT affects their deformability as given by their passage time (see §2.5.1).

We investigated two cell lines—one representing a spontaneous EMT that arose at the primary tumor site, and the other induced to undergo an EMT-like transition by direct contact with platelets (Fig. 2-1).

2.2 Spontaneous EMT*

The deformability change in a spontaneous EMT was measured in a murine tumor cell line derived from a mammary carcinoma of a Snail$^{YFP^+}_Y$/MMTV-PyMT animal. In this cell line, a YFP reporter is directly controlled by the endogenous promoter of the EMT-inducing transcription factor Snail, which allows fractionation of the cells according to Snail expression levels. This cell line was sorted using fluorescence-activated cell sorting (FACS) into an epithelial population (EpCAM$h^+YFP^+$) and a spontaneously arising mesenchymal (EpCAM$h^+YFP^+$) population. After sorting, the cells were propagated in monolayer culture for a few days, and differences in epithelial

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* This work was done in collaboration with Dr. Xin Ye of the laboratory of Prof. Robert A. Weinberg. Xin provided the cell line and performed FACS to sort the cells. Vivian C. Hecht of the Manalis Laboratory performed single-cell density measurements.
and mesenchymal morphologies were confirmed by phase-contrast microscopy (Fig. 2-2A). The two cell populations were then trypsinized and measured separately in the SMR.

![Diagram showing EMT process and measurement](image)

**Fig. 2-1.** Measuring the deformability of tumor cells undergoing an EMT
Carcinoma cells have been found to undergo a spontaneous EMT at the site of the primary tumor, and have also been found to undergo an EMT-like transition when in direct contact with platelets, which they encounter in circulation. Here, we measure tumor cells that undergo a spontaneous or platelet-induced EMT using an SMR with a constriction. (EMT = epithelial- mesenchymal transition, RBC = red blood cell, WBC = white blood cell, CTC = circulating tumor cell, EMT-like = epithelial-mesenchymal-like transition)

The measurement reveals that for cells of the same size, the cells of the mesenchymal population generally pass through the constriction faster than do those of the epithelial population (Fig. 2-2B). As previously described [59], lines were fit to each data set such that the difference in y-intercepts determines the ratio of the passage time of the mesenchymal population to that of the epithelial population, given the same cell size (Fig. 2-3, §2.5.2). In addition, for such close comparisons, buoyant mass values were converted to volume by measuring the density of the cells in the SMR (§2.5.1). On average, based on three replicates, the passage time ratio values indicate that the EpCAM<sup>lo</sup>YFP<sup>hi</sup> cells passed through the constriction 1.6-fold faster than the EpCAM<sup>hi</sup>YFP<sup>lo</sup> cells, for those having a volume greater than 1000 μm<sup>3</sup>. As expected, the EpCAM<sup>lo</sup>YFP<sup>hi</sup> population of cells was found to be more than two-orders of magnitude more tumorigenic than the EpCAM<sup>hi</sup>YFP<sup>lo</sup> when injected into mice (Ye and Weinberg, unpublished). This result is consistent with studies by other methods demonstrating that often, more mesenchymal or malignant cell types are more deformable [55,89].

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Fig. 2-2. Deformability changes in a spontaneous EMT
A) FACS sorted epithelial (EpCAM$^{\text{hi}}$YFP$^{\text{lo}}$) and mesenchymal (EpCAM$^{\text{lo}}$YFP$^{\text{hi}}$) populations of the MMTV-PyMT tumor cell line were cultured for two days to confirm differences in morphology prior to measurement in the SMR. Based on phase contrast microscopy, the mesenchymal population has decreased intercellular adhesion and is more elongated compared to the epithelial population, as expected. B) Passage time is plotted against volume, comparing the epithelial and mesenchymal subpopulations of the MMTV-PyMT cell line.
2.3 Platelet-induced EMT-like transition*

In addition to spontaneous EMTs, previous work has shown that direct signaling between platelets and cancer cells can induce the cells to undergo an EMT-like transition [24]. Thus, after one day of co-culture with platelets, carcinoma cells already exhibited increased expression of EMT-related, and prometastatic genes. Following an additional day of co-culture, carcinoma cells, having the majority of the platelets washed away during sample preparation, generated an increased number of lung metastases after tail-vein injection [24].

Using the same cell line and technique as published [24], Ep5 murine breast carcinoma cells were cultured with platelets for two days in order to induce the transition, as confirmed by decreased intercellular adhesion and elongated morphology (Fig. 2-4A). While one culture plate of cells was treated with platelets, two control plates were treated with an equal amount of buffer. Cells from all plates were then washed and measured separately in the SMR to compare any changes in deformability between the platelet-treated and buffer-treated cells, using the deformability difference between the two buffer-treated plates as a control (Fig. 2-4B). For each replicate, the platelet-treated cells took a longer time to pass through the constriction than control

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* This work was done in collaboration with Shahinoor Begum, who performed the platelet treatment of the cells and Prof. Richard O. Hynes, who provided guidance to the experimental design. The work was based on a manuscript from the laboratory of Prof. Hynes: Labelle, M., Begum, S., Hynes, R.O. “Direct signaling between platelets and cancer cells induces an epithelial-mesenchymal-like transition.” Cancer Cell. 2011;20:576-590. Vivian C. Hecht performed single-cell density measurements in the Manalis Laboratory.
Fig. 2-4. Deformability changes in a platelet-induced EMT-like transition in Ep5 cells
A) Phase contrast micrographs of control Ep5 cells (treated with buffer) and Ep5 cells treated with platelets. Cells were treated on day 0 after the images were taken. Subsequent images were taken on the following two days to observe morphological changes in the platelet-treated cells undergoing an EMT-like transition. B) Two control plates of Ep5 cells that had been treated with buffer were measured in the SMR. The difference in passage time versus volume between these two buffer-treated samples serves as a control for identifying significant differences in other cell line comparisons. C) SMR measurements of the epithelial buffer-treated Ep5 cells and the mesenchymal-like platelet-treated Ep5 cells after platelet incubation for two days.
cells (Fig. 2-4C). On average, for cells between 800 μm³ and 3500 μm³, the platelet-treated cells required a 1.5-fold longer time than the control cells to pass through the constriction.

In addition, to characterize the effect of direct contact with platelets on the MMTV-PyMT cell line that can spontaneously undergo an EMT (§2.2), the epithelial subpopulation (EpCAM<sup>hi</sup>YFP<sup>lo</sup>) was treated with platelets. After two days of co-incubation with platelets, the EpCAM<sup>hi</sup>YFP<sup>lo</sup> did in fact undergo a morphological change suggestive of an EMT-like transition, as they became more elongated and had somewhat decreased intercellular adhesion (Fig. 2-5A). Although in some replicates the platelet-treated cells had a slightly higher passage time than the buffer-treated cells, their change was not significantly different when compared to the passage time differences between two sets of buffer-treated control cells (Fig. 2-5B, C). Nonetheless, the passage time of the mesenchymal-like, platelet-treated population was certainly unlike that of the spontaneously arising mesenchymal population (EpCAM<sup>lo</sup>YFP<sup>hi</sup>, §2.2), in that the cells did not become more deformable than the founding epithelial population.

The passage time ratio of all replicates are plotted together for comparison in Fig. 2-6. Interestingly, different sets of control EpCAM<sup>hi</sup>YFP<sup>lo</sup> cells varied more from one another than did various sets of control Ep5 cells, possibly due to the composition of each cell type and the dynamic nature of the MMTV-PyMT cells that are known to undergo a spontaneous EMT or to technical variation. The deformability changes in tumor cells caused by signaling from platelet interactions have not been measured previously, and the potential decreased deformability brought about by this interaction differs from most studies referenced earlier that indicate that more malignant cells are often more deformable. Instead, as postulated by others, the decreased deformability or prevented increase in deformability may enhance hematogenous dissemination by augmenting survival under shear stress conditions [94,96,97]. In fact, the absolute values of the passage times for the Ep5 cells and the EpCAM<sup>hi</sup>YFP<sup>lo</sup> cells treated with platelets are not far from each other (Fig. 2-7), corroborating the idea that there may be an ideal stiffness, rather than a relative change in stiffness, for the cells to attain for survival in circulation [97]. Furthermore, a decrease in deformability or maintenance of a certain stiffness may increase the ability of the cells to lodge or be retained longer in microcirculation [25].
Fig. 2-5. Deformability changes in a platelet-induced EMT-like transition in EpCAM<sup>hi</sup>YFP<sup>lo</sup> cells
A) Phase contrast micrographs of control EpCAM<sup>hi</sup>YFP<sup>lo</sup> cells (treated with buffer) and EpCAM<sup>hi</sup>YFP<sup>lo</sup> cells treated with platelets. Cells were treated on day 0 after the images were taken. Subsequent images were taken on the following two days to observe morphological changes in the platelet-treated cells undergoing an EMT-like transition. B) Two control plates of EpCAM<sup>hi</sup>YFP<sup>lo</sup> cells that had been treated with buffer were measured in the SMR. The difference in passage time versus volume between these two buffer-treated samples serves as a control for identifying significant differences in other cell line comparisons. C) SMR measurements of the epithelial buffer-treated EpCAM<sup>hi</sup>YFP<sup>lo</sup> cells and the mesenchymal-like platelet-treated EpCAM<sup>hi</sup>YFP<sup>lo</sup> cells after platelet incubation for two days.
Fig. 2-6. Passage time ratios (mesenchymal:epithelial) for all replicates
Passage time ratios were calculated for three replicates of each control Ep5 experiment (different plates of buffer-treated Ep5 cells compared to one another: Control Ep5, No EMT), three replicates of the buffer-treated versus platelet-treated Ep5 comparisons (Platelet Ep5, EMT-like), three replicates of each control EpCAM<sub>hi</sub>YFP<sub>lo</sub> experiment (different plates of buffer-treated EpCAM<sub>hi</sub>YFP<sub>lo</sub> cells compared to one another: Control EpCAM<sub>hi</sub>YFP<sub>lo</sub>, No EMT), three replicates of the buffer-treated versus platelet-treated EpCAM<sub>hi</sub>YFP<sub>lo</sub> comparisons (Platelet EpCAM<sub>hi</sub>YFP<sub>lo</sub>, EMT-like), and three replicates of the MMTV-PyMT epithelial versus mesenchymal comparison (EpCAM<sub>lo</sub>YFP<sub>hi</sub>, Spontaneous EMT). Each passage time ratio represents the passage time of the mesenchymal population to that of the epithelial population. For the Ep5 cells, the population of cells having a larger volume (Fig. 2-4) is presumed to be doublets that did not dissociate after trypsinization, since those cells have twice the volume of the majority of cells, and hence were not included in the linear fits when determining the passage time ratio (§2.5.2). In comparing Ep5 platelet-treated to buffer-treated cells as well as in comparing EpCAM<sub>lo</sub>YFP<sub>hi</sub> to EpCAM<sub>hi</sub>YFP<sub>lo</sub> cells, the y-intercepts of the linear fits were found to be significantly different with p < 3.4x10<sup>-16</sup>. The error bars for each point corresponds to the 95% confidence interval of the passage time ratio based on the intercept offsets of the linear fits. The dark gray shaded regions are based on the extent of change seen in control experiments, and are symmetric regions drawn about a passage time ratio of 1.0, indicating no change in passage time characteristics for each given cell type.
Fig. 2-7. Passage time versus volume for platelet-treated Ep5 cells and platelet-treated EpCAM$^{hi}$YFP$^{lo}$ cells.

The passage time versus volume characteristics of platelet-treated Ep5 cells are similar to those of platelet-treated EpCAM$^{lo}$YFP$^{hi}$ cells. The data here are the same as in Fig. 2-4C and 2-5C.

2.4 Discussion

Based on the passage time measurements of single-cells, it is evident that cell deformability may change dynamically with molecular changes in an EMT. The measurements made on MMTV-PyMT cells that underwent a spontaneous EMT match previous literature showing that more malignant cells tend to be more deformable. However, we demonstrate here that Ep5 cells that were co-cultured with platelets and thus subject to TGFβ and NF-κB signaling, become less deformable after an EMT-like transition. Furthermore, the MMTV-PyMT cells that became more deformable in a spontaneous EMT, did not significantly alter their deformability when induced to undergo an EMT-like transition by direct contact with platelets. Although these findings are dissimilar to many previous studies, it does correspond to another study that suggests a wide range of tumor cell deformability, where a highly metastatic subpopulation of melanoma and renal cancer cells were shown to be stiffer than their less metastatic counterparts [94].

The fact that different mesenchymal cancer cell populations were found here to be more deformable, less deformable, or similarly deformable to their corresponding epithelial populations may relate to the extent of their EMT, especially since the cells undergo cytoskeletal reorganization during the phenotypic shift [98,99]. For example, the platelet-treated Ep5 and EpCAM$^{lo}$YFP$^{hi}$ cells may be undergoing an EMT-like transition, but have not reached a stage where they are as mesenchymal in phenotype as the spontaneously arising EpCAM$^{lo}$YFP$^{hi}$ MMTV-PyMT cells. Moreover, cells in different stages of the metastatic cascade may benefit from being stiffer or softer depending on the mechanism of their malignancy. More specifically, while a tumor cell is at the
primary site, becoming more deformable may allow it to better escape the tumor; whereas once a
tumor cell is already in circulation and binds to platelets, becoming stiffer or maintaining a certain
stiffness may enable the cell to attain to an optimal physical condition that resists damage in a
condition of shear stress, enhancing its viability in circulation [96, 97]. In addition, becoming less
deformable may augment the ability of tumor cells to lodge in a capillary at a distant site, or to be
retained longer at the site to allow for subsequent extravasation [24, 25, 94]. In fact, it has already
been shown that that platelet interaction with tumor cells does not necessarily increase in the initial
arrest of the cells, but does enhance metastatic tumor formation [24, 25, 94].

2.5 Materials and methods

2.5.1 Cell deformability and size measurements

An SMR having a 6 μm wide, 15 μm deep, and 50 μm long constriction was used to measure the
buoyant mass, as a metric for size, as well as the passage time, as a metric for deformability, of
each single cell as previously described (§1.2.2) [59]. The channel walls were coated with PEG (1
mg/mL; PLL(20)-g[3.5]-PEG(2); Surface Technology) for all experiments in this study. The
positively-charged PLL portion of the polymer adsorbs to the negatively-charged silicon dioxide
surface, while the inert PEG portion comes into contact with cells being measured. All SMR data
were processed in MATLAB as previously described [59].

In addition to deformability, the passage time measurement is also influenced by such
factors as cell size [59]. Therefore, in all analyses of deformability, cells of the same size were
compared. For measurements having small changes in passage times, such as in an EMT, the
buoyant mass of each cell was converted to volume, since the volume of the cell is more
consistently related to passage time than to buoyant mass. The single-cell densities of each cell
type were measured on each day of experiment using the SMR as previously described [100]. The
density of the culture medium in which the cells were measured was determined by the resonant
frequency of the SMR. Then, the volume of the cell was determined by the ratio of the buoyant
mass to the difference in the average cell density (typical interquartile range: 0.003-0.007 g/mL)
and the fluid density.

Moreover, the relative contribution of surface friction to the passage time can be accounted
for by the cell’s velocity as it transits through the constriction (transit velocity), after fully
deforming into its entrance [59]. However, the velocity of the cell upon entering the constriction
(entry velocity) is typically slower than the transit velocity, suggesting that the passage time is
mostly dictated by the time the cell spends deforming at the entrance of the constriction, rather
than transiting through afterwards (Fig. 2-8 A, B). The transit velocity of cells undergoing an EMT
was found to change by the same amount as the entry velocity, indicating that alterations in the
frictional component affect but do not dominate the differences in passage times (Fig. 2-8 C, D).
Hence, in the data presented here, we consider changes in passage times to represent changes in
deformability.
2.5.2 Calculating passage time ratio, removing secondary population of cells

The passage time ratios, as shown in Fig. 2-3 and Fig. 2-6, were calculated as previously described [59]. In brief, the passage time versus volume data were plotted on a log-log scale. A line was fit to each data set, having the same slope, but variable intercepts. The difference between the two intercepts corresponds to the log_{10} of the ratio of the passage times, which is then converted to the actual passage time ratio by exponentiation.

To obtain accurate linear fits, the data were considered within its linear region (on a log-log scale) containing the majority of the cells for analysis, eliminating small particles or debris. Thus, the lines were fit for Ep5 cells having volumes between 800 and 3500 \( \mu m^3 \), and for MMTV-PyMT cells greater than 1000 \( \mu m^3 \). As evident in Fig. 2-4 and noted in the caption Fig. 2-6, the Ep5 buffer-treated and platelet-treated cells have a second population of cells presumed to be doublets due to their being twice the volume of the majority of the cells in the population. To obtain accurate linear fits, the second population of cells was removed by excluding cells below a given line roughly parallel to the major axis of the second population of cells. The same line was used to remove the second population on both the platelet-treated and buffer-treated cells for all three replicates of the experiment. Fig. 2-9 shows an example of the removal of the larger population of cells.
Fig. 2-8. Entry and transit velocities
A) Entry velocity versus volume for EpCAM$^{\text{hi}}$YFP$^{\text{lo}}$ and EpCAM$^{\text{lo}}$YFP$^{\text{hi}}$ cells. B) Transit velocity versus volume for EpCAM$^{\text{hi}}$YFP$^{\text{lo}}$ and EpCAM$^{\text{lo}}$YFP$^{\text{hi}}$ cells. C) Entry and transit velocity ratios for each replicate of experiment are shown, comparing EpCAM$^{\text{hi}}$YFP$^{\text{lo}}$ to EpCAM$^{\text{lo}}$YFP$^{\text{hi}}$ as well as comparing Ep5 cells treated with platelets to those treated with buffer. The velocity ratios are calculated in the same manner as the passage time ratios described in the §2.5.2. The entry and transit velocities change similarly, with neither change dominating the other. D) For each measurement, the entry velocity ratio was divided by the transit velocity ratio to more clearly compare the contribution of each in both cell lines having undergone an EMT or EMT-like transition. There is no significant difference between the ratios for the two cases. Measurements made for each of these panels are the same as those presented in Fig. 2-2 and 2-4.
Fig. 2-9. Removal of putative doublets for passage time ratio analysis
As an example, the same data from Fig. 2-4C is shown here. To determine the passage time ratio between the two data sets, the shaded region was used to remove the second, larger population of cells, which was presumed to be doublets due to their size. The same shaded region was used to remove the secondary population of cells from all data sets with Ep5 cells.

2.5.3 Cell culture and preparation
The MMTV-PyMT tumor cell line was derived from a mammary carcinoma of a SnailYFP+/; MMTV-PyMT animal. The cells were cultivated in DMEM/F12 (1:1) (Invitrogen 11320) supplemented with 5% adult bovine serum (Sigma B9433), non-essential amino acids (Invitrogen 11140), and Pen/Strep (Invitrogen 15070). They were then stained with anti-EpCAM antibody directly conjugated to allophycocyanin (APC) fluorophore (Ebioscience 17-5791-82), and sorted by FACS based on EpCAM and SnailYFP expression. The cells were then cultured for 2 or 4 days (passaged once) and then trypsinized (0.08% Trypsin-EDTA), resuspended in culture medium, and measured in the SMR. Ep5 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM), 10% fetal calf serum (FCS), 1% penicillin/streptomycin and 2 mM L-glutamine and obtained as described previously [24]. Cells were detached from the plate using 0.25% Trypsin-EDTA. Cells were maintained in incubators at 37°C with 5% CO2.

2.5.4 Platelet preparation and treatment of cells
Platelets were prepared and Ep5 cells were co-cultured as previously described [24]. EpCAMhiYFPlo cells were co-cultured with platelets in the same way, but having 3% adult bovine serum in the medium since the cells would not survive without serum. EpCAMhiYFPlo cells were treated with platelets within a week of being sorted by FACS to ensure relatively high purity in the epithelial phenotype. Platelets were resuspended in PIPES buffer (1,4-
piperazinediethanesulfonic acid, Sigma-Aldrich), and at least 500 million platelets were used to treat each 6-cm dish of cells. Buffer-treated cells were treated with the same volume of PIPES buffer, but without platelets. When the cells were ready to be measured in the SMR, they were washed with PBS, trypsinized, and resuspended in culture medium. After washing and trypsinizing, the cells were expected to be largely free of bound platelets, as confirmed in a previous study [24].
Chapter 3

Passage Time Differences between Tumor Cells and Blood Cells in Circulation

3.1 Introduction

As tumor cells escape the primary tumor and intravasate into circulation, their environment changes entirely—from living with other carcinoma cells in a tissue to fluid flow conditions, where they endure shear stress and encounter narrow capillaries [1]. Thus, we measure the deformability of tumor cells, noting their distinctions from blood cells. We further investigate whether an environment like the circulatory system may passively select for certain types of cells based on their physical ability to deform and pass through a constricted space, as measured by an SMR (Fig. 3-1). Finally, we measure the deformability of circulating tumor cells CTCs from a limited number of cancer patients. Taken together, our results provide a physical perspective to complement and enrich the current understanding of the metastatic progression of carcinomas.

Fig. 3-1. Measuring the mechanical properties of tumor cells and blood cells in circulation
A schematic diagram demonstrating the context of circulating tumor cells and blood cells in the metastatic cascade as well as their measurement in an SMR with a constriction (EMT = epithelial-mesenchymal transition, RBC = red blood cell, WBC = white blood cell, CTC = circulating tumor cell, EMT-like = epithelial-mesenchymal-like transition)
3.2 Blood cells versus tumor cell lines

To begin probing the biophysical differences between cells that naturally live and survive in circulation versus ones that typically live within a tissue parenchyma, we first compared the passage time and buoyant mass properties of tumor cell lines versus blood cells. Fig. 3-2 shows distinct size and deformability characteristics between these two types of cells, where the small differences between mesenchymal and epithelial tumor cell phenotypes (Ep5 [Buffer-treated], Ep5 [platelet-treated], EpCAM<sup>hi</sup>YFP<sup>lo</sup>, EpCAM<sup>lo</sup>YFP<sup>hi</sup>, as presented in Chapter 2) pale in comparison to the dramatic differences between the tumor cell types and blood cells. Indeed, blood cells have passage times on the order of a few milliseconds, whereas tumor cells can have passage times as long as a few seconds. Included in the various tumor cell lines measured are a lung cancer cell line (H1975), two breast cancer lines (MDA-MB231 and SKBR-3), and a prostate cancer line (PC3-9). Meanwhile, the types of blood cells measured include human erythrocytes, peripheral blood mononuclear cells (PBMCs), polymorphonuclear (PMN) leukocytes, L1210 (mouse lymphoblast cell line), and primary mouse (BALB/c) leukocytes (§3.6).

As is already known, the majority of blood cells are smaller than most of the tumor cells in culture. However, even for cells of comparable size, as indicated by their buoyant mass, blood cells have decidedly faster passage times than do tumor cells. This distinction holds true for passage time measurements at two different flow rates, as portrayed by the similarity between Fig. 3-2A and B. For each flow condition, agglomerative clustering was employed on the median of the log<sub>10</sub> values of the passage times for binned buoyant masses, given a set range of buoyant masses where the different cell types overlapped (§3.6.2). The unsupervised algorithm demonstrates that the data naturally fall into two separate groups: tumor cells and blood cells (Fig. 3-2C, D). Moreover, when a lung cancer cell line (H1650) was spiked into enriched PBMCs, they maintained their distinction from the blood cells, where the number of cells having longer passage times corresponded to the number of tumor cells spiked into the sample (Fig. 3-3) [59]. It is also worth noting that both cell types were measured in a suspended state; thus even after trypsinization and resuspension in culture medium, the tumor cells maintain a marked physical distinction from blood cells. Given such a contrast, the question arises as to whether the circulatory system requires cells to possess a specific deformability characteristic in order for them to intravasate and remain in circulation.

3.3 Circulating tumor cells in a mouse model

To assess tumor cells circulating in vivo, we injected one million 4T1-ZSGreen (a murine mammary carcinoma line stably transfected to express ZSGreen) cells via tail vein into syngeneic BALB/c mouse hosts. 4T1 cells are known to express epithelial markers such as E-cadherin, while also expressing Twist, an EMT-related transcription factor [101,102]. In addition, they are known

* This work was done in collaboration with Shahinoor Begum, who cultured cells and performed all mouse work, in the laboratory of Prof. Richard O. Hynes, who provided guidance to the experimental design.
Fig. 3-2. Passage time and buoyant mass of blood cells versus epithelial and mesenchymal cell lines. Passage time versus buoyant mass plots demonstrate the distinct difference between tumor cell lines and blood cells, regardless of the epithelial or mesenchymal phenotype of the tumor cells. A) The same murine cell lines (Ep5, EpCAM<sup>hi</sup>YFP<sup>lo</sup>, EpCAM<sup>lo</sup>YFP<sup>hi</sup>) from Fig. 2-2 and 2-4 are shown here in comparison with blood cells: human red blood cells, human peripheral blood mononuclear cells (PBMCs), human polymorphonuclear (PMN) leukocytes, and a mouse lymphoblast cell line (L1210), measured under the same flow conditions (0.9 psi applied pressure). B) Four human cancer cell lines corresponding to lung (H1975), breast (MDA-MB-231, SKBR), and prostate cancers (PC3-9) compared to blood cells: human red blood cells, human PBMC, mouse white blood cells (WBCs) and L1210 cells, under faster flow conditions (1.5 psi applied pressure) portray that the same distinction in passage time profiles. For (A) and (B), the dotted grid lines on the (continued on the following page)
Fig. 3-2 continued: X-axis are at 10 pg and 100 pg, while dotted grid lines on the Y-axis are at 0.001 s, 0.01 s, 0.1 s, 1 s, 10 s, and 100 s. Also, the color of the scatter plots corresponds to the density of data points, with red being the highest and blue being the lowest density. C) The passage time data in (A) for buoyant masses between 30 pg and 150 pg are placed into 6 bins based on buoyant mass (log scale). The median of the log10 values of the passage times within each bin is shown in the heat map, where color assignment is on a log scale. The passage times are grouped using agglomerative clustering, with the dendrogram shown to the right of the heat map. The reported buoyant mass bin centers and passage time values are converted from log10 values. D) Similar to (C), the passage time data in (B) for buoyant masses between 30 pg and 180 pg are plotted in a heat map of passage time values, showing a dendrogram of the clustering to the right.

Fig. 3-3. H1650 cells spiked into a blood cell sample
A) SMR frequency signals showing some cells passing quickly, and others passing slowly through the constriction (§1.2.2). B) Passage time versus buoyant mass for blood cells (human PBMC, n = 2832), H1650 cells (n = 404), and blood cells spiked with H1650 cells (n = 10810). For the leukocytes spiked with H1650 cells, there is a subpopulation whose passage time and buoyant mass properties correspond to the H1650 cells alone. Measurements were acquired with an applied pressure of 1.5 psi. This figure was adapted from Ref. [59].

Tumor cells found to persist in the blood seven days after initial intravenous injection are expected to be cells that had detached from initially formed metastatic tumors and re-entered circulation, since the originally injected cells should have all been removed from circulation within seven days, if not within a few minutes [103,104]. In each of three replicates of the experiment, over 97% of the initially injected cells maintained in culture, spanned the same range of passage times as the tumor cells that were able to enter into the blood stream from the metastatic lesions.

Fig. 3-4A) Metastatic lesions formed in the lungs (Fig. 3-A4A) and blood was retrieved from the mouse via cardiac puncture. Tumor cells were then sorted from the blood using FACS, based on their ZSGreen expression and subsequently measured in the SMR. The measured cells from the blood were compared to the 4T1 cells that had been injected seven days prior (Fig. 3-4B, C), an aliquot of which had been kept in culture and run through FACS as a control (Fig. 3-5).

Tumor cells found to persist in the blood seven days after initial intravenous injection are expected to be cells that had detached from initially formed metastatic tumors and re-entered circulation, since the originally injected cells should have all been removed from circulation within seven days, if not within a few minutes [103,104]. In each of three replicates of the experiment, over 97% of the initially injected cells maintained in culture, spanned the same range of passage times as the tumor cells that were able to enter into the blood stream from the metastatic lesions.
In fact, for the one replicate having more than 40 CTCs in the size region of interest (40 pg to 120 pg), the distribution of passage times for CTCs is the same as that of the original 4T1 control cell line \( (p = 0.412, \text{Fig 3-4C}) \). Therefore, although the cells may transiently change their deformability during intravasation, our measurement reveals that there is not a strict deformability requirement that the cells must meet in order to enter circulation. Additionally, both the CTCs and the control cell line have higher median passage times (1.7- and 1.8-fold, respectively) than healthy BALB/c leukocytes, with the passage time distributions of the tumor cells being significantly different from that of the blood cells.

Due to the small number of CTCs in other replicates, rather than assessing the distribution of passage times, agglomerative clustering of median passage times was employed as in Fig. 3-2, combining the data from all replicates (Fig. 3-6A, B). As a result, the mouse CTCs cluster with the control 4T1 cells and not with the leukocytes (Fig. 3-6A). In addition, the CTCs also cluster with other murine cell lines, such as Ep5, EpCAM\(^{hi}\)YFP\(^{lo}\), EpCAM\(^{lo}\)YFP\(^{hi}\), and B16F10 melanoma cells (measured under the same flow conditions) instead of grouping with blood cells, including L1210, human PBMCs, and healthy BALB/c leukocytes (Fig. 3-6B). The data from individual replicates also separately cluster in the same fashion, grouping themselves with other tumor cells rather than blood cells (Fig. 3-6C, D). For reference, passage time versus buoyant mass scatter plots for the additional cell lines are shown in Fig. 3-7. Hence, cells that intravasate into the blood stream do not necessarily need to be as deformable as blood cells. It is noteworthy that based on the passage time characteristic alone, not taking into account any molecular differences, a cell having almost any measureable passage time from the original 4T1 population could intravasate into the blood stream, even though its passage time can be 10-fold higher than that of typical leukocytes.
**Fig. 3-4. Mouse CTC deformability**

A) Mouse lungs were imaged seven days after tail vein injection of one million 4T1-ZSGreen cells to confirm presence of metastatic lesions. B) Mouse blood, after lysing the erythrocytes, was run through FACS to sort out tumor cells having green fluorescence, and then measured in the SMR. The red triangles indicate each signal detected by the SMR while measuring CTCs. 4T1-ZSGreen control cells kept in culture after the initial injection were run through FACS for a comparison, and are shown as gray points in the background. C) The distributions of passage times for the 4T1-ZSGreen control cells, CTCs, and BALB/c leukocytes (same data as in Fig. 3-2B) are plotted for cells with a buoyant mass ranging from 40 pg to 120 pg, eliminating debris, aggregates, or stray blood cells sorted during FACS while focusing on cells large enough to interact with the constriction. The control cell line and the CTCs have similar passage time distributions ($p = 0.412$).
Fig. 3-5. FACS does not affect passage time characteristic of 4T1-ZSGreen cells
A) The effect of FACS on the passage time versus buoyant mass profile of 4T1-ZSGreen cells was determined by comparing an aliquot of cells that had been run through FACS and one that had not. B) A zoomed-in region of the data in (A), showing linear fits that were made to the two data sets. The passage time ratio between the cells after FACS and before FACS is 1.02, which is in the range of having no change as measured by the SMR (Fig. 2-6). The intercept offset between the linear fits to the two data sets is not significantly different (p = 0.233).
Fig. 3-6. Clustering analysis of mouse CTC passage times
A) Data from three replicates of the mouse CTC experiment are pooled to compare passage times in the given mass range. The heat map colors correspond to the passage time value, with the color assignments on a log scale. The binned passage time values were grouped by agglomerative clustering, with the dendrogram shown to the right of the heat map. B) CTC data from three replicates are pooled and compared to murine tumor cell lines other than the control 4T1 cell line, including Ep5, EpCAM<sup>hi</sup>YFP<sup>lo</sup>, EpCAM<sup>lo</sup>YFP<sup>hi</sup>, and B16F10, evaluated under the same flow conditions (1.5 psi applied pressure). Also included for comparison are the blood cells from Fig. 3-2B. The passage times are plotted in the heat map and the dendrogram from clustering analysis is shown on the right. C) Data from each replicate are used separately in the agglomerative clustering analysis. Each replicate clusters together with control 4T1 cells, and not with BALB/c leukocytes. D) Each replicate of the mouse CTCs clusters together with murine tumor cell lines other than 4T1, rather than blood cells. For all panels, the buoyant mass range was taken from 40 pg to 120 pg, and the reported buoyant mass bin centers and passage time values are converted from log<sub>10</sub> values.
Fig. 3-7. Passage time versus buoyant mass characteristics of murine tumor cell lines
The mouse tumor cell lines were measured with an applied pressure of 1.5 psi, which is the same condition as the measurements of mouse CTCs in the main text (Fig. 3-4). Plots are on a log-log scale, with an X-axis ranging from 3 pg to 600 pg, and a Y-axis ranging from 0.008 s to 300 s. Dotted grid lines on the X-axis are at 10 pg and 100 pg, while dotted grid lines on the Y-axis are at 0.001 s, 0.01 s, 0.1 s, 1 s, 10 s, and 100 s. Colors correspond to the density of data points, with red being the highest and blue being the lowest density.
3.4 Circulating tumor cells in metastatic prostate cancer patients*

We subsequently turned to human patient samples to understand whether tumor cells in cancer patients also maintain a passage time in the circulation distinct from that of blood cells. Blood samples from metastatic prostate cancer patients were processed with the CTC-iChip at the Massachusetts General Hospital, depleting the samples of the majority of red blood cells and white blood cells as previously described [44,45]. The two samples processed having a significant number of CTCs are presented here (Fig. 3-8). As expected due to residual blood cells in the samples, most of the cells passed through the SMR's constriction quickly, doing so without any apparent hindrance. However, to verify whether CTCs were also measured, all cells were collected from the output of the SMR and stained with DAPI for the nucleus, EpCAM to indicate a tumor cell, and counterstained for CD45 as a marker for leukocytes. To then determine whether there were CTCs that passed through the SMR’s constriction with a timescale comparable to that of blood cells (< 0.01 s), we compared the number of CTCs (DAPI positive, EpCAM positive, and CD45 negative) to the number of long passage time events (> 0.01 s). For both patients, there were more CTCs than long passage time events (1 more for Patient 1, 34 more for Patient 2), indicating that CTCs can behave like blood cells in terms of passage properties.

However, since it is known that CTCs can be small [31,44] and that most cells below 50 pg pass through the SMR’s constriction in less than 0.01 s regardless if they are deformable blood cells or potentially stiffer adherent cells placed into suspension (1.5 psi applied pressure, Fig. 3-2B), estimates were made of the buoyant mass of each CTC based on the imaged diameter (§3.6.8, Fig. 3-9 and Fig. 3-10). The buoyant mass of each CTC was not known directly since although they were measured by the SMR, the identity of each individual cell was not inherently known during the measurement. From the buoyant mass conversion, many CTCs were in fact small compared to the cells prepared from cultured cancer cell lines and measured previously (Fig. 3-2). For this reason, these CTCs were below the sensitivity range of the SMR to definitively compare their deformability to that of blood cells. However, there were some large CTCs that would be expected to have passage times greater than 0.01 s if they were mechanically similar to those deriving from a tumor cell line. Based on a combination of all the human tumor cell line data in Fig. 3-2B, a tumor cell between 50 pg and 100 pg has a probability of 0.58 of having a passage time greater than 0.01 s, and a tumor cell greater than 100 pg has a probability of 0.99 of having a passage time greater than 0.01 s. For Patient 1, there were 3 CTCs greater than 100 pg, while there was only one SMR measurement having a passage time greater than 0.01 s. If the CTCs are like

* This work was done in collaboration with Dr. Sangwon Byun in the Manalis Laboratory, who performed an equal share of work in measuring patient blood samples in a separate SMR, as we at times operated two different SMRs to measure the same sample. We also collaborated with Dr. David T. Miyamoto, Dr. Shannon L. Stott and Dr .Mehmet Tohner at the Massachusetts General Hospital (MGH). Dr. Stott helped to direct the study, enabled the acquisition of patient blood samples, and provided guidance for identifying CTCs by immunofluorescence staining. Dr. Miyamoto also aided in experimental design, coordinating the acquisition of metastatic prostate cancer blood samples and providing guidance in sample preparation based on his prostate cancer expertise. Katherine Broderick and Woosel Kim, also at MGH, performed blood sample preparation in the CTC-iChip prior to SMR measurement.
Fig. 3-8. Human patient CTC deformability
A) SMR measurement of prostate cancer Patient 1 blood sample, after having been processed in the CTC-iChip [44,45]. B) Based on fluorescence images, the diameter of each CTC was converted to an approximate buoyant mass value to visualize where they fall among the data measured by the SMR as shown in (A). C) A sampling of fluorescence images of CTCs and a white blood cell (WBC) taken from the same prostate cancer patient sample after it was measured in the SMR. D) SMR measurement of prostate cancer Patient 2 blood sample, after having been processed in the CTC-iChip. E) Based on fluorescence images, the diameter of each CTC was converted to an approximate buoyant mass value. F) A sampling of fluorescence images of CTCs and a WBC from the same patient sample. False color overlays were applied for composite fluorescence images.
the tumor cell lines, the probability of 2 of the 3 CTCs having passage time below 0.01 s is 0.0003. Similarly, for Patient 2, there were 5 CTCs between 50 and 100pg, and the probability of all of them having passage times faster than 0.01 s to match the SMR measurements is 0.013. Thus, it is unlikely that all of the larger CTCs in these patient samples are physically similar to the tumor cell lines for cells of the same size; rather many of them more closely resembled blood cells in their ability to pass through the constriction quickly.

**Fig. 3-9.** CTC diameters
CTC diameters were determined as the maximum Feret’s diameter of the EpCAM fluorescence signal for each cell.
The density distribution of PC3 cells measured by the SMR was used to convert CTC diameters to buoyant masses.

3.5 Discussion

In considering tumor cells in circulation, we have found that in spite of deformability changes due to an EMT, tumor cells in general (whether having an epithelial or mesenchymal phenotype) have much longer passage times than blood cells. However, based on the mouse model presented, there does not seem to be a specific deformability necessary for the tumor cells to exit the initially formed metastases and enter circulation. More specifically, the tumor cells that enter circulation from the metastatic lesions are not particularly more deformable than the originally injected population of tumor cells. This observation is consistent with the fact that during invasion and intravasation, the surrounding extracellular matrix can be proteolytically degraded [105] and endothelial cell junctions may be weakened [106], possibly allowing tumor cells to pass through without requiring them to be especially deformable.

In human prostate cancer patients, however, the throughput limitations of the SMR device required patients to have high CTC levels. Based on two patient samples having CTCs, many of their CTCs were able to pass through the constriction in less than 0.01 s, as fast as blood cells. Part of this phenomenon is due to the small size of many CTCs. However, if CTCs were mechanically similar to the various tumor cell lines we studied, it would be unlikely for the larger CTCs to also have a fast passage time as was seen in the SMR measurements. Thus, while the mouse model demonstrates that blood-cell-like deformability is not necessary for tumor cells to intravasate and enter circulation, limited human patient samples show that some CTCs in circulation may in fact

* Single-cell density data were acquired by Vivian Hecht in the Manalis Laboratory.

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find it advantageous to be small in size or to be as flexible as blood cells to rapidly pass through a constricted channel.

Based on these findings, one conceivable hypothesis regarding metastasis could be that many tumor cells having a broad range of biophysical characteristics intravasate into circulation, but only a small (potentially more deformable) subpopulation persists in circulation, while others lodge at distant sites for metastasis. Another possibility is that the primary tumors of different patients may be quite disparate and may evolve both in the size and deformability of their cells, shedding CTCs that are representative of biophysical changes in the primary tumor. Certainly, the limited data presented here would need to be much expanded and developed to verify such hypotheses. However, due to the low throughput of the SMR (45 µL/hr) combined with the rarity of CTCs, further enhancements to the platform would need to be implemented to make a more complete study feasible. In addition, based on the SMR measurements in total, there may exist a few relatively stiff CTCs that take a long time to pass through a constriction; however, it would be necessary to isolate cells with long passage time events from the SMR device in real-time to be definitive. A new method developed for this purpose will be discussed in the following chapter (Chapter 4), and data from Patient 2 will be revisited. Fully recognizing the limitations in the presented study, here we demonstrate an initial biophysical measurement of clinical samples revealing the ability of some prostate cancer CTCs to pass through a narrow constriction quickly either by being small in size or by becoming as deformable as blood cells. Future modifications to the SMR platform, such as increased throughput via parallelization as well as smaller constriction sizes, may be able to further probe the deformability of CTCs and more definitively define whether they are in fact mechanically indistinguishable from blood cells.

3.6 Materials and methods

3.6.1 Single cell passage time and size measurements

A suspended microchannel resonator (SMR) having a 6 µm wide, 15 µm deep, and 50 µm long constriction was used to measure the buoyant mass, as a metric for size, as well as the passage time, as a metric for deformability, of each single cell as previously described (§1.2.2) [59]. The channel walls were coated with PEG (1 mg/mL; PLL(20)-g[3.5]-PEG(2); Surface Technology) for all experiments in this study. The positively-charged PLL portion of the polymer adsorbs to the negatively-charged silicon dioxide surface, while the inert PEG portion comes into contact with cells being measured. All SMR data were processed in MATLAB as previously described [59], and passage time density scatter plots were created using a modified version of the dscatter function (MathWorks File Exchange) [72]. Here, due to the large distinction in passage times between blood cells and tumor cells, along with the unfeasibility of measuring the single-cell density of a cell type as rare as CTCs, buoyant mass was used as a metric for cell size, and was not converted to volume.
3.6.2 Cluster analysis of passage time data

For data compared by clustering analysis, log_{10} values of the buoyant mass and passage time data were used, both in binning buoyant mass values and determining median passage times. Thus, the passage time value reported for each buoyant mass bin is the median of the log_{10} values of the passage times converted back to an actual time by taking it as an exponent of 10. The reported buoyant mass bin centers were also converted from log_{10} values by exponentiation. The agglomerative hierarchical clustering analysis was performed using MATLAB, and plots were created using the clustergram function.

3.6.3 Cell culture and preparation

Details concerning Ep5, EpCAM^{hi}YFP^{lo}, and EpCAM^{lo}YFP^{hi} cell lines are delineated in §2.5.3. B16F10 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM), 10% fetal calf serum (FCS), 1% penicillin/streptomycin and 2 mM L-glutamine. 4T1 cells were stably transfected to express ZSGreen as previously described [107], and were cultured in DMEM, 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 2 mM L-glutamine, 1% non-essential amino acids. MDA-MB231, SKBR3, and PC3-9 cell lines were obtained and cultured as described previously [44]. H1975 and H1650 cells were obtained previously [108], and cultured in RPMI supplemented with 10% FBS, 1% sodium pyruvate, and 100 IU of penicillin and 100 μg/mL streptomycin. L1210 cells (a gift from Dr. Marc Kirschner) were maintained in suspension in RPMI with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 25 mM HEPES. Cells were maintained in incubators at 37°C with 5% CO2.

3.6.4 Blood cell preparation

Whole human blood was purchased from Research Blood Components, LLC (Brighton, MA). Mononuclear cells were separated using Histopaque-1077 (Sigma-Aldrich 10771). Polymorphonuclear lymphocytes were enriched by using Lympholyte-poly (Cedarlane CL5070). Red blood cells were measured by diluting whole blood in PBS with 1% (w/v) Kolliphor P188 (Sigma-Aldrich), since the concentration of red blood cells is orders of magnitude higher than that of other cell types. The Kolliphor nonionic copolymer-based buffer reduces nonspecific cell attachment to tubing and channel walls [45].

Healthy BALB/c mouse blood was obtained via cardiac puncture. Red blood cells were removed by lysis (150 mM NH4Cl (8.02 g/L), 10 mM KHCO3 (1.00 g/L), 0.1 mM Na2EDTA, pH 7.23) or Histopaque-1077. To isolate live leukocytes and ensure removal of clots and platelets, the remaining cell solution was stained for CD45 (Biolegend 103121) and CD41 (Biolegend 101319). DAPI (Life Technologies D1306) was also used to stain dead cells. After resuspending the cells in PBS with 0.5% FBS and 2 mM EDTA, cells were sorted using fluorescence-activated cell sorting (FACS) and the CD45^{hi}/CD41^{lo}/DAPI^{lo} cells were collected for analysis in the SMR.
3.6.5 Mouse CTC generation and preparation

Balb/c mice were injected via tail vein with 1 million 4T1-ZSGreen cells in 100 μL of Hank’s Balanced Salt Solution (HBSS, Life Technologies). After seven days, blood was collected by cardiac puncture (left ventricle) into 400 μL of citrate dextrose solution (ACD; 38 mM citric acid, 75 mM trisodium citrate, 100 mM dextrose). Fluorescent images were taken of the lung to verify tumor formation. Meanwhile, red blood cells were lysed using lysis buffer for 2 minutes on ice. Cells were then resuspended in phosphate buffered saline (PBS) with 0.2% FBS and prepared for FACS. Cells were collected into culture medium, filtered with a 70 μm mesh, and subsequently measured in the SMR.

3.6.6 Human patient CTC SMR measurements and staining

Patients with a diagnosis of prostate cancer provided informed consent to an Institutional Review Board approved protocol (DF/HCC 05-300) to allow donation of blood for this study. Patient blood samples were prepared at the Massachusetts General Hospital (MGH), where negative depletion was performed using the CTC-iChip as previously described [44,45]. Samples were maintained at room temperature in PBS with 1% Kolliphor P188 during SMR measurement. For Patient 1, measured cells were placed in a 24-well glass bottom plate coated with Cell-tak (BD Biosciences 354240). Paraformaldehyde (PFA) was then added to each well to reach a final concentration of 4% in each well (PFA was made fresh from 16% PFA, Electron Microscopy Sciences 15710). The well-plate was then spun at 821 rcf for 10 min to ensure adherence of the cells. After over 30 min of fixation, the cells were permeabilized with 1% Igepal (Sigma-Aldrich), rinsed, and incubated with 3% bovine serum albumin (BSA, Fisher) and 5% mouse serum (Jackson ImmunoResearch 015-000-120) in PBS. Cells were then stained with anti-Cadherin-11 and anti-EpCAM antibodies both conjugated to Alexa Fluor 488 (R&D Systems FAB17901G, Cell Signaling Technologies 5198S), anti-CD45 conjugated to PE-CF594 (BD Biosciences 562279), and DAPI (Life Technologies D1306). The cells were then rinsed and stored in PBS for fluorescence imaging. For Patient 2, after passing through the CTC-iChip, cells were stained with anti-EpCAM (Cell Signaling Technologies 7139) and anti-CD45 (Invitrogen MHCD4520) antibodies. Stained, live cells were then measured in the SMR and collected in a 24 well plate. PFA was added to a final concentration of 4%, along with DAPI (1.43 μM final concentration) for nuclear staining. Based on the concentration of the samples during negative depletion, an equivalent of approximately 1 mL of blood from Patient 1 was measured, and an equivalent of 1.6 mL of blood from Patient 2 was measured in the SMR.

3.6.7 Fluorescence imaging of CTCs

Fluorescence imaging was done on a Nikon Ti inverted microscope (Swanson Biotechnology Center Microscopy Core Facility). All imaging of CTCs was performed at 40x magnification. Images were taken on a Photometrics CoolSnap HQ camera. The diameter of each cell was found
by taking the maximum Feret’s diameter of the fluorescence EpCAM signal after background subtraction using ImageJ.

### 3.6.8 Converting imaged cell diameter to buoyant mass

The PC3 prostate cancer cell line was used to estimate the single-cell density of prostate cancer CTCs. PC3 cells were stained for EpCAM and resuspended in PBS with 1% Kolliphor for SMR measurement as would typically be done with patient samples. The single-cell densities of one aliquot of cells were measured in the SMR, while another aliquot was placed in a 24-well plate where they were fixed in 4% paraformaldehyde (PFA) and imaged. From image processing, we found that the maximum Feret’s diameter determined by ImageJ software corresponded well to the volume measured by the SMR. The maximum Feret’s diameter found for each CTC is shown in Fig. 3-9. Since the number of CTCs was low and the density distribution of the PC3 cells in 1% Kolliphor buffer was found to be quite spread compared to typical density measurements (interquartile range: 0.017 g/mL, Fig. 3-10), instead of using one set density value to convert all of the CTC diameters to buoyant mass, a simulation was used to randomly assign a density value from the measured distribution to each CTC. Buoyant mass was then calculated by the following equation:

\[
\text{Buoyant mass} = \frac{4}{3} \pi R^3 (\rho_{\text{cell}} - \rho_{\text{fluid}}),
\]

where \( R \) corresponds to the radius of the imaged cell, \( \rho_{\text{cell}} \) is the density of the cell, and \( \rho_{\text{fluid}} \) is the density of the buffer used for measurement. The result is plotted in Fig. 3-8B and 3-8E. To determine the number of cells that fell in the buoyant mass range of 50 pg to 100 pg, or greater than 100 pg, the simulation was repeated 1000 times. The average number of cells in each buoyant mass range of interest was calculated and reported in the main text.
Chapter 4

Biophysical-Molecular Correlation with the Suspended Microchannel Resonator*

4.1 Introduction

Mechanical properties of single cells have drawn increasing attention in their ability to identify changes in cell phenotype, including those in differentiation and malignancy, and even to provide diagnostic value [55,56,58–60]. Various techniques used to probe the deformability of single cells include micropipette aspiration [57,74], atomic force microscopy [62,63], microrheology [65–68], optical stretching [70,71], hydrodynamic deformation [72,73], and microfluidic constriction devices [59,75–77]. One such microfluidic device is the suspended microchannel resonator (SMR), which, based on the resonance frequency of the sensor, records the position of the center of mass of a cell with up to sub-micron precision as it passes through the constriction (§1.2.2, Fig. 4-1A, B). The resonance frequency signal enables the measurement of the passage time of a cell through the constriction, and can be used to parse out such information as the relative contributions of deformability and surface friction [59]. In addition to the position of the cell, the resonance frequency changes are indicative of the buoyant mass of a cell, as a metric of its size [59].

In measuring biophysical properties of cells with an unknown identity, it is vitally important to have molecular validation, not only to identify the cells being measured, but to gain insight into deeper molecular underpinnings of the biophysical observations and the cell to cell variation. Although some precise single-cell measurement techniques lend themselves more easily than others to optical imaging simultaneously with the biophysical measurement [72,92,109–115] until recently, few of these methods have demonstrated the ability to isolate particular cells of interest for downstream molecular study [116]. Meanwhile, passive sorting and bulk filtration methods have been developed, where a general population of cells is collected based on its deformability [42,117–121]. Examples include mechanical filters, microfluidic margination, and inertial microfluidic devices [42,117–121]. However, using these techniques, precise single-cell deformability properties remain unknown. The variation in molecular characteristics cannot be correlated to the deformability metric of each cell.

* This work was done in collaboration with Dr. Sangwon Byun, who helped with SMR measurements of healthy donor and patient blood samples. LabVIEW code used for cell detection was adapted based on code originally written by Nathan Cermak in the Manalis Laboratory. Dr. David T. Miyamoto, Dr. Shannon L. Stott, and Dr. Mehmet Toner at the Massachusetts General Hospital helped coordinate acquisition of healthy donor and patient blood samples, and provided guidance for the biological aspects of the study. Katherine Broderick and Wooseok Kim helped to process blood samples in the CTC-iChip prior to SMR measurement.
Here, we present a cell sorting method for the SMR to correlate the single-cell biophysical measurement with the molecular expression of each cell of interest. In particular, we demonstrate this technique in characterizing tumor cells in blood, with a view to better understand the physical properties of circulating tumor cells (CTCs). Although previous studies indicate that epithelial cancer cell lines tend to take longer to pass through constrictions than do blood cells (see §3.2) [41,59], the rarity of CTCs has caused studies regarding the physical properties of actual CTCs to remain sparse [53,95]. Nonetheless, a technique such as the CTC-iChip, which concentrates CTCs by eliminating the majority of erythrocytes and leukocytes, enables the SMR to measure CTCs in spite of its limited throughput (~45μL/hr). Using this combination of techniques, some cancer patient blood samples were found to have more cells with long passage times (> 10ms) than did healthy donor blood samples. However, long passage time events may be created by debris, aggregates, or atypical blood cells within the sample. As a proof of principle, we address this limitation in measuring tumor cells in processed blood samples, by collecting each long passage...
time event off-chip and correlating the biophysical SMR measurement to immunofluorescence images.

4.2 Materials and methods

4.2.1 Suspended microchannel resonator for buoyant mass and passage time measurements

As described in §1.2.2, a suspended microchannel resonator (SMR) having a 6 μm wide, 15 μm deep, and 50 μm long constriction was used to measure the buoyant mass (as a metric for size) and passage time (as a metric for deformability) of single cells [59]. The resonance frequency of the cantilever sensor, determined by the buoyant mass and position of the cell, is recorded as each cell passes through the sensor (Fig. 4-1A, B). This enables the assessment of the shape of each cell’s signal, including the cell’s velocity as it deforms into the entrance and when it transits through the constriction ([§1.2.2]) [59].

All data analysis was performed in MATLAB as described previously [59], using the dscatter function (MathWorks File Exchange) for Fig. 4-2 [72]. The position of the cells in the cantilever sensor used in Fig. 4-5, were calculated as described previously, where a value of -1 corresponds to the tip and 0 corresponds to the base of the cantilever ([§1.2.2]) [59,83]. Also, normalized time is the time recorded by the sensor divided by the total length of time the cell takes to pass through it, such that time 0 is when the cell is at the tip of the cantilever and time 1 is when the cell exits the cantilever sensor.

4.2.2 Cell culture

H1650 cells were obtained and cultured as previously described [31]. PC3 and LNCaP cells were obtained from the American Type Culture Collection. LNCaP cells were cultured in RPMI (1640) with 10% fetal bovine serum (FBS). PC3 cells were cultured in DMEM/F-12 medium containing 10% FBS.

4.2.3 Blood cell preparation for initial test

Human blood was purchased from Research Blood Components, LLC (Brighton, MA). Mononuclear cells were enriched using Histopaque-1077 (Sigma-Aldrich) and resuspended in 1% (w/v) Kolliphor P188 (Sigma-Aldrich) in phosphate buffered saline (PBS). For the H1650-spiked sample, H1650 cells were added to the enriched mononuclear leukocytes, resulting in a final concentration of 600,000 cells/mL. The sample was then stained with anti-Cadherin-11 and anti-EpCAM antibodies both conjugated to Alexa Fluor 488 (R&D Systems FAB17901G, Cell Signaling Technologies 5198S), and anti-CD45 conjugated to PE-CF594 (BD Biosciences 562279).
4.2.4 Healthy donor and cancer patient blood samples processed in CTC-iChip and stained prior to SMR measurement

Healthy donor blood was obtained from healthy volunteers under an Institutional Review Board (IRB) approved protocol. Patients having a diagnosis of prostate cancer provided informed consent to a separate IRB-approved protocol (DF/HCC 05-300) to allow blood donation for the study. Blood samples were processed through the CTC-iChip in negative selection mode at the Massachusetts General Hospital (MGH), as previously described [44,45]. Briefly, the CTC-iChip removes most of the erythrocytes and leukocytes from the blood, leaving an enriched tumor cell sample. For LNCaP- and PC3-spiked samples, the cultured cells were placed into whole blood prior to processing in the CTC-iChip. After tumor cell enrichment, spiked samples and patient samples (other than Patient 3) were then stained with anti-EpCAM (Cell Signaling Technologies 7139) and anti-CD45 (Invitrogen MHCD4520) antibodies. Stained, live samples were then measured in the SMR, while maintained at room temperature in 1% (w/v) Kolliphor P188 in PBS.

4.2.5 Cell sorting method for SMR

Software was written in LABVIEW to determine the passage time of each cell in real-time, during transit through the SMR. For passage times greater than a user-defined threshold of 10 ms, the computer-controlled pressure regulators automatically stops the flow in the device (Fig. 4-1C).* For Figure 4-3B only, images of the cells were taken in the exit channel of the SMR immediately after the flow was halted. After successful cell capture was validated, it was deemed no longer necessary to image cells in the SMR output channel. The output tubing is then flushed with 1% Kolliphor in PBS into a 96-well plate, collecting the cells in a 50 µL volume. The cells having fast passage times that did not trigger a collection response were all collected in a 24-well plate for imaging together at the end of the experiment. After collection from the SMR, paraformaldehyde (PFA, made fresh from 16% PFA, Electron Microscopy Sciences 15710) was added to a final concentration of 4%, along with DAPI (Life Technologies D1306, 1.43 µM final concentration) for nuclear staining.

4.2.6 Fluorescence imaging

For immunofluorescence imaging in the output channel of the SMR, images were acquired on an Edmund Optics monochrome CMOS camera (EO-1312M). After cell collection fluorescence imaging was carried out on a Nikon Ti inverted microscope at either 20x or 40x magnification (Swanson Biotechnology Center Microscopy Core Facility). The EpCAM expression intensity levels were quantified from 40x images. Since the depth of field is small (~1 µm), the fluorescence

* The software has been updated to trigger the flow to stop based on a user-defined range of buoyant masses (frequency shifts) and a user-defined range of passage times simultaneously. However, this functionality has not yet been fully tested.
intensity values were normalized by the cross-sectional area of each cell. For future biological studies, a larger depth of field or deconvolution microscopy (involving images of multiple z-sections of the cell) should be used to capture the fluorescence expression of the entire cell. Images were acquired using a Photometrics CoolSnap HQ camera, and images were processed using ImageJ.

4.3 Results and discussion

Healthy donor as well as metastatic cancer patient blood samples were measured in an SMR after processing in the CTC-iChip, in order to identify whether there was a difference between their passage time and buoyant mass characteristics, and if so, to specify a gating region for cell collection (Fig. 4-2) [44]. In general, the healthy donor blood cells had passage times faster than 10 ms, except in some cases where a few smaller cells (< 50 pg) had longer passage times (> 10 ms) possibly due to debris or stickiness from platelet aggregation (Table 4-1). Cancer patient blood samples were prepared in the same manner and compared to healthy donor samples (Fig. 4-2B). Since it was notable that some patient samples had cells with longer passage times, it was of interest to see whether the cells that were larger than 50 pg and had passage times longer than 10 ms were in fact CTCs. Hence, we developed and implemented the cell sorting technique to collect cells having longer passage times and classify each cell based on its surface protein expression.

First, to validate the cell sorting technique, H1650 cells (human lung cancer cell line) were spiked into enriched mononuclear blood cells and stained for EpCAM/CDH11 to denote tumor cells, and CD45 to counterstain for leukocytes. The sample was then measured in the SMR (Fig. 4-3A). The flow in the device was automatically halted by computer-controlled pressure regulators when a long passage time was detected by custom software. To ensure that each long passage time event was indeed triggered by a tumor cell, once the flow was paused in the device, the cell was imaged in the output channel of the SMR prior off-chip collection (Fig. 4-3B). Due to the thickness and curvature of the glass on the SMR’s exit channel, only low quality images were obtained, but the staining was apparent and verified that each of the long passage times were caused by individual tumor cells. Then, to obtain higher quality images and to provide functionality for future downstream applications, each cell that had been imaged in the device was subsequently sorted off-chip into a separate well of a 96-well plate, with each well having a final volume of 50 μL. As revealed by fluorescence microscopy of the well plate, one tumor cell was found in each well, indicating that each tumor cell seen in the SMR channel was successfully captured off-chip (Fig. 4-3C). Note that in addition to the individual tumor cells, there were some leukocytes in a few of the wells, due to the fact that if some leukocytes pass through the constriction just prior to the tumor cell and have not exited the tubing of the device, they may be flushed into the 96-well plate together with the detected tumor cell. Thus, the purity of the collection depends upon the concentration of cells in the sample, which can be adjusted if higher purity is necessary. In many applications, an enrichment of the tumor cells may be sufficient, such as to establish a correlation between the molecular staining of the tumor cell to its biophysical passage time and buoyant mass.
signature. These results demonstrate that we can successfully collect cells based on specific physical properties and observe their particular molecular expression.

Fig. 4-2. Healthy donor versus metastatic prostate cancer patient blood sample
A) Passage time vs. buoyant mass measurements of a healthy donor blood sample, after having been depleted of most of the erythrocytes and leukocytes. An equivalent volume of ~280 μL of blood was measured. B) Passage time vs. buoyant mass measurements of a metastatic prostate cancer patient blood sample, having been depleted of most of the erythrocytes and leukocytes. An equivalent volume of ~360 μL of blood was measured. The difference in the number of cells measured was due to different efficiencies of the preprocessing steps in depleting the erythrocytes and leukocytes from the samples (§4.2.4). Colors correspond to the density of data points, with red being the highest and blue being the lowest density.
Table 4-1. Healthy donor blood samples measured in an SMR

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Blood Volume (µL)</th>
<th>Equivalent Total Number of SMR Peaks</th>
<th>Number of SMR Peaks with Passage Time &gt; 0.01s</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>All</td>
</tr>
<tr>
<td>Healthy 1</td>
<td>360</td>
<td>2791</td>
<td>0</td>
</tr>
<tr>
<td>Healthy 2</td>
<td>200</td>
<td>4688</td>
<td>0</td>
</tr>
<tr>
<td>Healthy 3</td>
<td>280</td>
<td>5509</td>
<td>0</td>
</tr>
<tr>
<td>Healthy 4</td>
<td>400</td>
<td>3261</td>
<td>2</td>
</tr>
<tr>
<td>Healthy 5</td>
<td>400</td>
<td>15977</td>
<td>1</td>
</tr>
</tbody>
</table>

A) SMR Measurement
Triggering Cell Collection

B) Images Taken in SMR Output Channel
Prior to Collection in Well-Plate

C) Fluorescence Microscopy After Cell Collection

<table>
<thead>
<tr>
<th>Cell Counts</th>
<th>Well 1</th>
<th>Well 2</th>
<th>Well 3</th>
<th>Well 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>EpCAM/CDH11 only</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>CD45 only</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

Fig. 4-3. Validation of tumor cell detection and collection in 96-well plate

A) Passage time vs. buoyant mass measurements of H1650 cells spiked into enriched mononuclear blood cells. The cells included in the boxed region (> 50pg buoyant mass & > 0.01 s passage time) were imaged on-chip as well as collected off-chip for additional verification. B) Images taken on-chip, in the exit channel of the SMR, immediately after custom software detected a long passage time signal. It was confirmed that all detected signals falling into the region of interest corresponded to a tumor cell by immunofluorescence imaging. C) Each detected H1650 cell was successfully collected off-chip in a 96-well plate. Based on additional immunofluorescence imaging of each well, a tumor cell was identified in each well (EpCAM/CDH11+ and CD45−), along with a few leukocytes (CD45+ and EpCAM/CDH11−).
Next, we further tested the sorting method by characterizing human prostate cancer cells (LNCaP and PC3) spiked into whole blood. The spiked samples were processed through the CTC-iChip to enrich for tumor cells (§4.2.4), stained for cell surface markers EpCAM and CD45, and measured in the SMR (Fig. 4-4). Cells that had passage times longer than 10 ms were sorted into a 96-well plate. Although we particularly focused on cells larger than 50 pg, a few of the smaller cells causing long passage times were also collected for image analysis. As listed in Table 4-2, for the spiked LNCaP cells, four of the five wells collected in the region of interest corresponded to a LNCaP cell. For the spiked PC3 cells, seven out of the nine wells collected corresponded to at least one PC3 cell. Two of the wells contained two PC3 cells, possibly because one of the two had a faster passage time due to being smaller in size or to being more deformable. In both cell lines, there were some tumor cells having passage times faster than 10 ms, and were not collected since they could not be clearly distinguished from blood cells.

Moreover, this technique enables the details of each SMR signal to be related to the protein expression in each cell. Thus, not only the passage time and buoyant mass, but the resonant frequency response denoting the position of the cell’s center of mass is recorded, as dictated by the biophysical properties of the cell as it passes through the sensor (Fig. 4-5A, §1.2.2, §4.2.1). It is interesting to note the relationship between the SMR peak shapes to the imaged cells (Fig. 4-5B). For both wells L2 and L3, corresponding to LNCaP cells, the signal obtained from the SMR corresponds to the canonical shape for a tumor cell (Fig. 4-1B) [59]. In contrast, wells L5 and L6 corresponding to debris, had SMR peak shapes that look markedly different, where the particle spends most of the time adjusting itself to go through the entrance of the constriction. It is possible that debris is introduced into the sample during the various pre-processing steps. Last, well L7 contained an activated neutrophil, and its corresponding SMR peak shape had distinct features of the cell passing through the constriction, unlike other cells having the same buoyant mass. Perhaps this may be indicative of the polymorphonuclear shape of the nucleus altering its position as it transits through the constriction as well as altering its buoyant mass.

Indeed, hierarchical clustering analysis of the positions of the measured cells or particles as they pass through the constriction over time (normalized, §1.2.2, §4.2.1) demonstrates that particles of similar nature, as identified by fluorescence imaging, can cluster together by the shapes of their SMR signals (Fig. 4-5C,D). The signals corresponding to LNCaP cells cluster together, whereas the signals corresponding to debris cluster separately, and the activated neutrophil falls in between. Although a more thorough and systematic study would need to be done to identify the causes of each particular detail of the SMR peak shape, the newly developed method relating each peak to a corresponding cell may help elucidate such details. Cataloging such SMR signals may eventually allow us to identify particles being measured in the device without the need for downstream imaging.
Fig. 4-4. Cell line-spiked blood samples
A) A blood sample spiked with LNCaP cells was measured in the SMR. The labeled measurement events were collected off-chip (L1-L7). B) A blood sample spiked with PC3 cells was measured in the SMR. The labeled measurement events were collected off-chip (P1-P9). In both panels, the dotted area corresponds to the region of interest (> 50 pg and > 0.01 s).
Table 4-2. Collected measurement events from cell line-spiked blood samples

<table>
<thead>
<tr>
<th>Spiked Cells</th>
<th>Well Label of Collected Peak</th>
<th># Cells</th>
<th>Well Content Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNCaP</td>
<td>L1</td>
<td>2</td>
<td>1 DAPI^−CD45^−EpCAM^+ (LNCaP)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 DAPI^−CD45^+EpCAM^− (Unidentified WBC)</td>
</tr>
<tr>
<td></td>
<td>L2</td>
<td>1</td>
<td>DAPI^−CD45^−EpCAM^+ (LNCaP)</td>
</tr>
<tr>
<td></td>
<td>L3</td>
<td>1</td>
<td>DAPI^−CD45^−EpCAM^+ (LNCaP)</td>
</tr>
<tr>
<td></td>
<td>L4</td>
<td>1</td>
<td>DAPI^−CD45^−EpCAM^+ (LNCaP)</td>
</tr>
<tr>
<td></td>
<td>L5</td>
<td>0</td>
<td>Debris (Not in region of interest)</td>
</tr>
<tr>
<td></td>
<td>L6</td>
<td>0</td>
<td>Debris (Not in region of interest)</td>
</tr>
<tr>
<td></td>
<td>L7</td>
<td>1</td>
<td>DAPI^−CD45^−EpCAM^− (Activated Neutrophil)</td>
</tr>
<tr>
<td>PC3</td>
<td>P1</td>
<td>1</td>
<td>DAPI^−CD45^−EpCAM^+ (PC3)</td>
</tr>
<tr>
<td></td>
<td>P2</td>
<td>2</td>
<td>2 DAPI^−CD45^−EpCAM^+ (PC3)</td>
</tr>
<tr>
<td></td>
<td>P3</td>
<td>1</td>
<td>DAPI^−CD45^−EpCAM^+ (PC3)</td>
</tr>
<tr>
<td></td>
<td>P4</td>
<td>0</td>
<td>Debris</td>
</tr>
<tr>
<td></td>
<td>P5</td>
<td>1</td>
<td>DAPI^−CD45^−EpCAM^+ (PC3)</td>
</tr>
<tr>
<td></td>
<td>P6</td>
<td>1</td>
<td>DAPI^−CD45^−EpCAM^+ (PC3)</td>
</tr>
<tr>
<td></td>
<td>P7</td>
<td>1</td>
<td>DAPI^−CD45^−EpCAM^+</td>
</tr>
<tr>
<td></td>
<td>P8</td>
<td>0</td>
<td>Debris</td>
</tr>
<tr>
<td></td>
<td>P9</td>
<td>2</td>
<td>2 DAPI^−CD45^−EpCAM^+ (PC3)</td>
</tr>
</tbody>
</table>

Note that for cells to be positive for CD45 or EpCAM, the cell had to have a clear ring of fluorescence, indicating surface expression of the protein. If no clear ring of fluorescence was present at the boundary of the cell, general fluorescence was considered to be background, non-specific fluorescence. WBC = white blood cell.
Fig. 4-5. SMR peak shape correlation with immunofluorescence staining

A) Raw SMR frequency signal versus time acquired for each measurement event, demonstrating different peak shapes for different types of cells or particles being measured. Labels correspond to those in (B) as well as in Fig. 4-4A. Note that the axes are different for each peak for better visualization of the details of each peak shape. B) Immunofluorescence images of cells corresponding different peak shapes from (A), with L2 and L3 being LNCaP cells, L7 being an activated neutrophil, and WBC being a typical white blood cell. Note that the intensity of EpCAM for L3 was reduced to avoid saturation, as it was much more intense than all other imaged cells. C) The position of the cell or particle in the SMR as it passes through the constriction (-1 corresponds to the tip, and 0 corresponds to the base of the cantilever) is plotted versus normalized time (0 corresponds to when the cell reaches the tip of the cantilever and 1 corresponds to the time the cell exits from the cantilever) for each of the SMR signals that were collected for visual analysis. D) The median positions of each measured signal within each bin of normalized time are plotted as a heat map. A dendrogram derived from hierarchical clustering analysis is shown to the right of the heat map, demonstrating that the shape of the SMR signals can be categorized into different groups, potentially related to the type of particle measured, as identified by fluorescence imaging.
Furthermore, this technique also allows us to compare the relative expression of surface proteins of each cell to their passage times and buoyant masses. As a proof of principle, H1650 cells that were stained for EpCAM were sorted into separate wells of a 96-well plate and imaged. The fluorescence images were quantified as an indication of relative levels of EpCAM expression (Fig. 4-6). Thus, the additional dimension of single-cell relative surface protein expression level can be added to each single-cell biophysical SMR measurement. Such relationships may provide meaningful information regarding the correlation between molecular and physical traits of single cells as well as the differences between subtypes of cells (such as more mesenchymal or more malignant) within a given cell population.

Finally, to test the biophysical-molecular correlation method on primary patient samples, a limited number of metastatic prostate cancer patient blood samples (n = 7) were measured, following the same protocol. Five samples were determined to have no CTCs. Of the two samples that were confirmed to contain CTCs, only one sample (Patient 4) had a cell in the gating region of interest (Fig. 4-7). This cell was collected and was found in fact to be a CTC based on its EpCAM+CD45−DAPI− signature, indicating that there can be CTCs in circulation that are stiff, having long passage times. The rest of the measured sample was collected and pooled together in a 24-well plate at the end of the measurement and no CTC was found. Interestingly, as discussed in Chapter 3, there are other cases in which some CTCs in circulation have passage times faster than 10 ms. Patient 2 in Chapter 3 was assessed using the sorting method presented here, and was found to have 35 CTCs with fast passage times (<10 ms) and none with long passage times. However, this method has enabled us to validate that in some patient blood samples, long passage times greater than 50 pg may still indeed correspond to CTCs.
Fig. 4-7. Metastatic prostate cancer patient blood sample
A) Passage time versus buoyant mass measurements of a metastatic prostate cancer patient blood sample. An equivalent total of 1.8 mL of blood was measured. B) One cell that fell in the region of interest that corresponded to a tumor cell-like frequency signal in the SMR as well was collected off-chip and imaged. Fluorescence images indicate that the SMR measurement correlated to a CTC (EpCAM positive, CD45 negative). Images of a white blood cell (WBC) from the remainder of the sample is shown for comparison.

4.4 Conclusion

We have demonstrated the proof-of-principle that we can detect and collect individual cells of interest after measurement in the SMR, using tumor cell-spiked blood samples as well as a metastatic prostate cancer patient sample. After collection, we performed immunofluorescence imaging on the cells and correlated them to the biophysical measurement made by the SMR. Not only did we measure the passage time and buoyant mass of each cell, but the SMR signal shape held additional information in identifying the type of cell or contaminating particle in the sample. In addition to identifying the types of cells being measured, we provide evidence that the SMR measurement can be correlated to molecular signatures such as relative EpCAM expression measured by fluorescence microscopy. Finally, we demonstrated the ability to identify and collect a CTC based on its stiff mechanical properties relative to its surrounding blood cells. Although there is no clinical relevance to this study since it is based on one patient sample, high throughput systems that exploit arrays of SMR may enable clinical studies that utilize mechanical biomarkers based on cell size and passage time. Nevertheless, the examples presented here demonstrate the ability to correlate SMR measurements to molecular information from individual cells. Because cells can now be sorted off-chip based on their physical characteristics, in the future this technique can be extended for other downstream assays, including analysis of DNA or RNA of cells of interest. Moreover, as the SMR platform has already demonstrated the ability to measure multiple physical parameters, the technique may be developed further to collect cells based on their buoyant mass, growth rate, or density [78,100,122]. The demonstrated cell sorting technique, correlating
biophysical to molecular properties, will enable future studies to elucidate how physical changes relate to specific fundamental molecular changes within single cells.
Chapter 5

Additional Project: A Microfluidic “Baby Machine” for Cell Synchronization*

5.1 Abstract

Common techniques used to synchronize eukaryotic cells in the cell cycle often impose metabolic stress on the cells or physically select for size rather than age. To address these deficiencies, a minimally perturbing method known as the “baby machine” was developed previously. In the technique, suspension cells are attached to a membrane, and as the cells divide, the newborn cells are eluted to produce a synchronous population of cells in the G1 phase of the cell cycle. However, the existing “baby machine” is only suitable for cells which can be chemically attached to a surface. Here, we present a microfluidic “baby machine” in which cells are held onto a surface by pressure differences rather than chemical attachment. As a result, our method can in principle be used to synchronize a variety cell types, including cells which may have weak or unknown surface attachment chemistries. We validate our microfluidic “baby machine” by using it to produce a synchronous population of newborn L1210 mouse lymphocytic leukemia cells in G1 phase.

5.2 Introduction

Synchronization of cells in the cell cycle is imperative in the study of many cellular and molecular processes. It specifically allows for the study of molecular regulatory mechanisms and genetic expression throughout the cell cycle, which has facilitated the discovery of biomarkers for cancer prognosis and drug [123–125]. In addition, since cells have different molecular compositions and distinct responses to stimuli while in different phases of the cell cycle, synchronization methods are used to reduce biological noise in experiments involving populations of cells.

The most common techniques for synchronizing eukaryotic cells involve the addition or depletion of certain compounds that stall the cells at a particular phase in the cell cycle. The cells are then released to continue progression of the division cycle by the removal of the drug or re-addition of growth factors. For example, in serum starvation, removing serum from a growing culture can cause the cells to enter a quiescent state, and upon the reintroduction of serum, the cells reenter the cell division cycle. However, serum starvation has been found to reduce cell survival and increase DNA fragmentation, and reentry into the cell cycle is often not synchronous.

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Similarly, DNA synthesis inhibitors such as excess thymidine and hydroxyurea stall cells in S phase, while microtubule polymerization inhibitors such as nocodazole and colcemid stall cells in mitosis\[128\]. Nonetheless, such compounds have unwanted side-effects, including activating the DNA damage response, influencing RNA synthesis, and inducing apoptosis, especially at higher concentrations [129–133]. Although these batch chemical methods may align cells to a certain feature of the cell cycle, it is known that such drugs perturb the growth and progression of internal molecular machinery. For instance, even while DNA synthesis is inhibited, other cellular processes such as transcription and translation still continue, creating a growth imbalance in the cell [134]. Thus, mitotic and replication inhibitors place cells in nonphysiological states, and even though synchronization with them may be valid for some experiments, the extent of their usefulness may be limited [127,135–137]. The resulting growth imbalance may particularly confound studies regarding cell growth, metabolism, and their relation to the cell cycle. Indeed, it has even been found that protein expression in chemically synchronized cells does not match that of unperturbed cells [138]. Hence, the applicability of results from cells obtained by batch chemical methods to normal, unperturbed cells remains questionable.

Another common way to synchronize cells is physically extracting a subpopulation of cells with similar properties, rather than coercing the entire culture to converge at the chosen characteristic. One such technique is counterflow centrifugal elutriation, which uses centrifugal force balanced by a centripetal flow of fluid in a specially designed centrifuge rotor to separate the cells by their sedimentation velocity, which is dependent upon cell size and shape [139–141]. Although this method successfully isolates different sizes of cells, with the smallest cells assumed to be in G1 phase, it exposes them to harsh conditions, such as a 683 x g rotor speed at 20°C for 4-5 hours [141]. More recently, cells have been separated by size in microfluidic devices using dielectrophoresis, acoustophoresis, hydrophoresis, hydrodynamic filtration, or inertial forces [142–146]. However, the size of the cell does not necessarily indicate its age, so the resulting similarly-sized cells may still have a range of ages due to inherent biological variation. Cells may also be sorted based upon DNA content using flow cytometry, but the use of DNA-staining dyes may cause mutagenicity and affect cell [128,134]. Furthermore, a synchronous batch of G1 cells selected based on DNA content rather than a particular event in the cell cycle could have an age range spanning the length of G1, which can occupy over 40% of the eukaryotic cell cycle, depending on the cell type [147]. In addition, both centrifugal elutriation and flow cytometry require costly equipment.

A simpler and less perturbing method for synchronizing cells is mitotic shake-off. Adherent cells that grow in a monolayer culture often become spherical during mitosis, at which point they can be detached by shaking or agitation. Since mitosis comprises only a short portion of the cell cycle, sometimes the shake-off method is used in conjunction with chemical synchronization methods to increase yields; this introduces the same concerns mentioned previously with chemical techniques [129,148,149]. Moreover, the mitotic shake-off method is only possible for adherent, monolayer cell cultures [128,134].
In the 1960s, Helmstetter et al. developed the membrane elution method of synchronization, colloquially known as the "baby machine" [150]. In this method, cells are bound to a membrane, and as they divide, one cell remains adhered while the other is eluted. This yields a population of cells synchronized at the beginning of G1 phase. The technique was first used with bacteria, and was later customized for mammalian cells in 2002 and improved upon in 2006 [150–152]. Although the “baby machine” is arguably the least perturbing method of cell synchronization, it has not been widely adopted. Furthermore, the technique depends on the ability of cells to bind to membranes coated with concanavalin A, poly-D-lysine, etc.[151]. Cells with weak or unknown binding ability would thus be difficult or impossible to synchronize using the “baby machine.”

Here we present a technique in which a pressure differential is used to adhere thousands of cells to a surface, while allowing for constant perfusion of culture medium as well as elution of newborn cells. This technique requires no specific cell surface chemistry and should be amenable to virtually any suspension cell type. As a proof-of-principle, we fabricated and tested microfluidic devices that include an array of 2-μm diameter trapping holes onto which cells can be loaded. We demonstrate that L1210 mouse lymphocytic leukemia cells load into the device, grow and divide on chip, and elute a synchronous population of cells in G1 phase.

5.3 Materials and methods

5.3.1 Device description

The silicon and glass microfluidic “baby machine” consists of an introduction channel that is bifurcated twice and feeds into the main cell capture chamber. This chamber is 4 mm wide and 5 mm long, and houses 2109 trapping holes onto which cells can be captured (Fig. 5-A). The holes are staggered and spaced 45 μm from one another, and are placed at least 1.75 mm away from the entrances and exits of the chamber to allow a uniform flow profile to develop. The dimensions and spacing were chosen to minimize the variation in pressures and stresses experienced by the cells in different regions of the capture chamber. The small pressure drop horizontally across the chamber also allows the vertical trapping pressures to be relatively uniform and sensitively controlled for optimal operation.

Four access ports provide fluidic access to the device: inlet, bypass, backside, and outlet. The inlet is where fluid is introduced, the bypass allows for quick rinsing away of excess cells, the backside enables control of the vertical pressure drop across the trapping holes, and the outlet is where the eluted newborn cells are collected. An external fluid valve placed in-line with the bypass tubing is opened during rinsing and closed during perfusion. Although separate pressure regulators can manipulate the pressure at each port, we found that successful operation of the device could be achieved by using only one regulator at the inlet, while leaving the other ports at atmospheric pressure. Finer pressure balances were achieved by adjusting tubing resistances and fluid levels in
the vials. These fine-tuned parameters generally remain unchanged from one experiment to the next.

Fig. 5-1. Synchronization device design and operation
A) Image of the synchronization device illuminated from the backside, showing branching fluidic channels that lead into a cell capture chamber with 2109 2-μm diameter holes onto which cells can be loaded. In the schematic diagram, the side view is an illustration of the cross section along the dotted line in the top view. The channels are etched into the top glass wafer, and the 2 μm holes and fluidic ports are etched into the bottom SOI wafer. B) Operation of the device is controlled entirely by pressure differences. A positive pressure applied at the inlet creates both a pressure differential in the vertical direction across the holes to capture cells, as well as a pressure drop in the horizontal direction to generate lateral flow for the collection of daughter cells. An asynchronous population of cells is fed into the device and captured onto the holes, and with a constant slow perfusion of cell culture medium, the cells grow within the device. As they divide, the newborn cells, which are not anchored to trapping holes, are carried away in the fluid flow and eluted into a collection chamber. The eluted cells then compose a synchronous population of cells that has just completed cytokinesis and entered the G1 phase of the cell division cycle.

5.3.2 Device fabrication
The devices were fabricated using standard silicon and glass microfabrication techniques. The top layer consists of a 500-μm thick, 6-inch glass wafer (Bullen), with fluidic channels etched into it at two depths: 80 μm for the cell capture chamber and 30 μm for all other channels. The bottom of the devices is a 6-inch silicon on insulator (SOI) wafer (Ultrasil Corporation), having a 15 μm device layer and a 0.5-2 μm thick buried oxide layer. The 2-μm diameter trapping holes as well as the fluidic access ports were etched through the device layer using deep reactive ion etching (DRIE). After patterning the backside, the SOI was mounted onto a dummy wafer and DRIE was used to etch a reservoir below the trapping holes and open up the fluidic access ports from the
The exposed oxide was then stripped in 25% HF to fully release the trapping holes and fluidic access ports. Finally, the glass and silicon wafers were anodically bonded.

### 5.3.3 System assembly

The silicon-glass device is set in a polytetrafluoroethylene (PTFE) gasket with perfluoroelastomer o-rings, while the entire setting is clamped between two aluminum plates to create a fluidic seal. The aluminum plates have openings cut out for microscope viewing from above and fluidic connections from below. The aluminum clamp then resides in a copper setting that enables temperature control via a recirculating water bath (Thermo Scientific). In all experiments, the temperature of the device was maintained at 37°C.

To introduce fluid into the device, computer-controlled electronic pressure regulators (Proportion Air) were used to pressurize glass vials to which the fluidic tubing was connected through PTFE septa. The tubing that connected the vials to the device were made of fluorinated ethylene propylene (IDEX Health and Science), and were of varying inner diameters (0.15 mm - 0.41 mm), chosen to balance the resistances of the fluid flow. The outlet tubing was also kept short to minimize transit time for the cell to leave the device and enter the collection chamber.

### 5.3.4 Device operation

In preparation for cell synchronization, the device is first filled with ethanol to ensure flow through all of the trapping holes and to rid the system of air bubbles. Next, it is rinsed with purified, filtered water (Milli-Q) for at least one hour with an input pressure of 37 kPa; this cleans the surface and purges any remaining air bubbles. To reduce surface fouling, the channels are then treated with poly(L-lysine)-graft-poly(ethylene glycol) co-polymer (Surface Technology, Switzerland) at a concentration of approximately 0.5 mg/mL in phosphate buffered saline (PBS) for 30 minutes. The device is again rinsed with water and then primed with fresh cell culture medium at 37°C.

Next, to load cells into the device, the pressure regulator at the inlet is set to 3.4 kPa. After the cells are loaded, fresh, pre-warmed media is placed at the inlet and the excess cells are rinsed away. Then, the pressure at the inlet is reduced to approximately 0.6 kPa; the resulting lateral flow rate perfuses nutrients to the cells while imparting a minimum amount of shear stress. Using a plane Poiseuille flow approximation in the main chamber, the highest shear stress experienced by the cells during the transient loading of the device is 0.02 Pa (0.2 dyne/cm²), while the shear stress during long term perfusion is 0.003 Pa (0.03 dyne/cm²); both of these values are well below physiological shear stresses [153,154]. The perfusion rate is 40 μL/hr and the flow in the device is laminar (Re ≈ 0.003-0.02 in the main chamber).

To ensure that insecurely docked cells do not detach from the device and contaminate the output, the collection of cells does not begin until after an hour of perfusion. For the experiments presented here, we collected a new fraction of cells in a fixative every 12 hours to validate the count and synchrony of the output. Since the cells are fixed upon exiting the device,
the synchrony of the output is maintained for the duration of cell collection. However, in an experiment where one wished to use the live, synchronized G1 cells outputted by the device, the collection duration would be dictated by the desired number of cells and degree of synchrony: a short collection would yield a smaller number of cells with very similar ages, and a longer collection would yield a larger number of cells with increased age variability. Afterwards, the device can be washed using 10% bleach or piranha (3:1 [v/v] H2SO4:H2O2), rinsed thoroughly with water, and reused.

5.3.5 Cell count and cell cycle analysis

To assay the throughput and synchrony of the output from the device, cells were eluted into a formaldehyde fixative solution and, at 12-hour intervals, were counted and stained with DAPI. Since the fixative solution becomes more dilute as the cells elute, the initial solution contained 6% formaldehyde (Sigma-Aldrich F1635) in 1 x PBS, such that the final concentration of formaldehyde after 12 hours of collection would be 4%. For every 12-hour fraction of fixed output from the device, the number of cells in a 40-μL portion was counted manually using bright field microscopy. The determined concentration was then used to extrapolate the total number of cells eluted over the 12-hour period.

After a portion of the eluted cells were counted, the rest were stained using 3 μM DAPI (Invitrogen D1306) with 0.1% Triton X-100 (Sigma-Aldrich T-8787). Following 20 minutes of incubation with the dye, the cell solution was centrifuged at 300 x g for 6 minutes and resuspended in 20 μL of 1 x PBS. For the data presented, the cells were imaged on a glass slide using a 10x objective (Nikon, CFI Plan Fluor) on an upright Nikon microscope. To validate this method, replicate experiments were imaged using deconvolution fluorescence microscopy (enhanced additive method) with a 20x objective (Olympus, UAp340) on an Applied Precision DeltaVision Spectris Imaging System, which yielded similar results (not shown) [155]. The process of obtaining the fluorescence intensities of the cells in each image was automated using a macro script for ImageJ. The results were then analyzed using scripts written in MATLAB and further validated manually to ensure that only cells, and not dust or debris, were being used as data.

5.3.6 Cell culture

L1210 mouse lymphocytic leukemia cells were cultured in Leibovitz L-15 medium (Invitrogen 21083027) supplemented with 10% fetal bovine serum (Invitrogen 16000), 0.4% glucose (Sigma-Aldrich G8769) and 1% penicillin/streptomycin (Invitrogen 151400). They were grown in 25 cm2 culture flasks at 37°C in an atmosphere containing 5% carbon dioxide. Cells were seeded at a concentration of around 5 x 104 cells/mL and diluted into fresh medium every two days. The concentration of cells used during experiments was between 2 x 105 and 4 x 105 cells/mL.
5.4 Results and discussion

5.4.1 Working principles

The synchronization device is designed such that pressures alone control both the adherence of cells onto the device and the elution of G1 cells out from the device. Similar to the original “baby machine,” an asynchronous population of cells is loaded into the device, and a synchronous population of newborn cells is collected at the outlet. However, as shown in Fig. 1b, instead of using surface chemistries to trap cells, the microfluidic “baby machine” captures cells by the application of a vertical pressure differential across 2 μm diameter trapping holes. With a constant slow perfusion of cell culture medium in the horizontal direction, the cells grow within the device. When the cells divide, the newborn cells that are not anchored to a trapping hole are carried away by the fluid flow and eluted into a collection chamber. The eluted cells compose a synchronous population of cells that has just completed cytokinesis and has entered the G1 phase of the cell division cycle.

5.4.2 Cell loading and viability

We first verified that the cells could be loaded onto the trapping holes without being squeezed through or sheared off of them during the constant perfusion of culture medium. L1210 cells were introduced into the device and observed via microscopy. Bright field images (Fig. 5-2A) show that the cells can indeed fill the device, with most holes trapping a single cell. Since the loading mechanism is an active pressure differential rather than a passive settling probability, a large fraction (60% - 80%) of the imaged holes is occupied by cells after rinsing away insecurely-captured cells. In addition, the fraction of occupied holes generally increases over time as newborn cells are caught onto empty neighboring holes (Fig. 5-2B). However, not all of the holes end up capturing a cell because some may be obstructed by debris. The steady fraction of loaded holes achieved over time indicates that cells rarely come unstuck from the holes, preventing contamination of the eluted cells with non-newborn cells.

The loaded cells then remain on the device to grow and divide over time, as shown in Fig. 5-3A [see Supplemental movie http://www.rsc.org/suppdata/lc/c2/c2lc40277g/c2lc40277g.avi]. Although only a subset of images is shown, cells were observed to grow and divide on chip for the complete duration of the experiment (3 days). Next, to assess the health of the cells growing in the device, the mean interdivision time of the cells seen in time-lapse images was compared to the cell doubling time in culture. The interdivision time was taken as the time from the beginning of one cytokinesis to the beginning of the next cytokinesis. Figures 5-3B and C display the interdivision times of two consecutive division cycles for cells docked on the trapping holes. The mean interdivision times of 10.8 hours (between the first and second divisions) and 10.6 hours (between the second and third divisions) are comparable to the 10-11 hour doubling time observed in culture, and also agree well with the times given in previous studies [151,156]. The reduction in the reported number of second interdivision times is mainly due to some cells dividing while not being
imaged, and other cells slipping through the trapping holes either because of the pliability of the cells [examined more carefully in Chapters 1-4] or differences in hole sizes caused by variations in the microfabrication process. The results indicate that cells can grow and divide well on chip under the constant perfusion of media.

Fig. 5-2. Loading cells onto the device
A) Bright field image of a section of the main channel loaded with L1210 cells. Shown here are 245 cells on 304 holes (80% loaded). B) Fraction of occupied holes over time. Initially, after insecurely-trapped cells are rinsed away, 64% of the imaged holes are occupied by cells. Over time, however, newborn cells are captured by nearby empty holes, such that eventually over 90% of the holes are occupied.
Fig. 5-3. Cells growing and dividing on the device
A) Time-lapse images of five cells growing and dividing on the device. The time denoted below each image indicates elapsed time after the device had been loaded with cells and rinsed to remove any unattached cells. Fluid flow is from left to right. Colored dotted circles track cytokinesis followed by separation and release of a newly divided cell. Note that the bottom left cell (circled in blue) divides twice within the given time frame. B) Distribution of the first interdivision time of cells growing in the device, counted from the initial signs of pinching during the first division to the initial signs of pinching during the second division, as captured by time-lapse images (5 min./frame). N = 150 cells, median = 10.8 hrs, mean = 10.9 hrs, standard dev. = 1.5 hrs. B) Distribution of the second interdivision time, taken as the time between the second and third division using the same method as in A). N = 135 cells, median = 10.4 hrs, mean = 10.6 hrs, standard dev. = 1.3 hrs. The mean interdivision times through both divisions correspond well to the observed doubling time of the cells grown in bulk culture (10-11 hrs).
5.4.3 Yield and synchrony

After validating cell growth in the device, the output of the microfluidic "baby machine" was assessed to determine the yield and synchrony of the eluted cells. Figure 4a depicts the number of cells collected at each 12-hour time point. The initial increase in cell count between the first two time points corresponds with the fact that not all of the trapping holes are filled with cells initially, and as the trapped cells divide, some of the newborn cells are caught onto nearby vacant holes rather than being eluted from the device (Fig. 5-2B). Then, from 24 to 36 hours, the number of cells outputted plateaus at ~1000 cells every 12 hours. Based on the number of cells in the device and their doubling time, the output may be lower than expected. Although not affecting the health of the cells, causes for lower yield may include imprecision in estimating the total number of cells on the device and in the collection chamber, as well as loss of cells in the gasket interface. Furthermore, some cells slip through the trapping holes after having been docked, allowing newborn cells to occupy the recently vacated holes, which then lowers the overall yield of the device. In spite of this, the output reaches a steady value and the cells are not only growing in the device, but also dividing and producing new cells for at least 36 hours of device operation.

Also at each 12-hour time point, the fixed fractions of eluted cells were stained with DAPI to evaluate the distribution of DNA content. In Fig. 5-4B, the cells' fluorescence intensities were normalized such that the G1 DNA content distribution has a modal value of 1.0 unit. Based on the distribution of the control data taken from an asynchronous population of cells, a threshold was set at 1.2 units, corresponding to one standard deviation above the mean of a Gaussian curve fitted to the G1 peak. Cells with a fluorescence intensity less than this value (indicated by the shaded regions in Fig. 5-4B) are considered to be in G1 phase, while values above the threshold correspond to cells in S/G2/M. The G2/M peak falls at an intensity less than 2.0 units, most likely due to chromatin packing during G2 and formaldehyde induced cross-linking that may have hindered the access of the dye to DNA binding sites [157]. For an asynchronous, exponentially-growing control population, 52% of the cells have a DAPI intensity of less than 1.2 units (and thus are in G1). Within the first 36 hours of operation, a purity of up to 83% of the cells having G1 DNA content was obtained from our microfluidic "baby machine."

Next, a Kolmogorov-Smirnov test was carried out to compare the fraction at each time point to the control population. The null hypothesis was rejected each time, having p-values of 0.026, 9.1x10-10, and 1.8x10-4 at the 12-, 24-, and 36-hour time points, respectively. Hence, the cells being outputted from the microfluidic "baby machine" have a significantly different DNA content distribution than the asynchronous control population. From the overlaid kernel density estimates shown in Fig. 5-4C, it is clear that at all of the time points the probability density of the G1 peak increased, while the G2/M shoulder diminished compared to the asynchronous population, suggesting a largely synchronous population of cells in G1.
Fig. 5-4. Yield and synchrony
A) Yield from the device over time. Synchronized cells were eluted into a formaldehyde fixative and fractions were collected and counted every 12 hours. Note that the system produces a steady output from 24 to 36 hours. B) DAPI fluorescence intensity distributions for each 12-hour fraction. The control population is an asynchronous population of cells taken from bulk culture. At each time point a threshold of 1.2 fluorescence intensity units is set to estimate the fraction of cells corresponding to G1 DNA content, as indicated by the percentages written in each shaded region. N = 677, 41, 128, 144 cells, for the asynchronous, 12-, 24-, and 36-hour time points, respectively. C) Kernel density estimates of DAPI intensity distributions for each time point, overlaid with that of the asynchronous control. The dotted vertical line marks the 1.2 unit threshold. In all cases, the fluorescence intensity data are normalized such that the G1 modal value is at 1.0 units. The right shoulder in the control data, corresponding to DNA content of cells in G2/M, is clearly diminished for the fractions taken at the output of the device. Also note the increased probability density of the G1 peaks of the device output (dashed lines) compared to the asynchronous population (solid line), indicating synchrony in G1.
Additional information regarding the age of the cells was garnered from bright field time-lapse images. The elapsed time from the appearance of a cleavage furrow during cytokinesis until the cells fully separate and one disappears from the field of view was calculated, and the distribution of these times is shown in Fig. 5-5. The median amount of time for cells to separate is 1.6 hours. Note that this time includes the time for cytokinesis. Here, 83% of the cells separated within 4 hours of beginning cytokinesis. This means that at least 83% of cells were collected in G1 phase, since G1 phase (not including cytokinesis time) for L1210 cells should be 4-5 hours according to data reported in literature [158]. The similarity between the fraction of G1 cells estimated by separation time and that obtained by DAPI staining indicates that the purity of synchrony is partly affected by inherent biological variation in cytokinesis and cell separation. This variation would also affect previous baby machines, which have been shown to maintain synchrony over many divisions [151]. In summary, according to both DAPI staining and separation times calculated from time-lapse imaging, over 80% of the eluted cells are in G1 phase.

![Fig. 5-5. Cell separation time](image)

Time for a cell to separate from its sister cell after morphological signs of a cleavage furrow are evident, taken from time lapse images (5 min./frame). N = 150 cells, median = 1.6 hrs, mean = 2.2 hrs, standard dev. = 2.0 hrs. 83% of the cells fully detached from its sister cell within 4 hours, including time for cytokinesis.

### 5.5 Conclusions

The presented cell synchronization device serves to demonstrate the principle of a pressure-controlled baby machine. Rather than using surface chemistries to adhere cells to a membrane, the microfluidic device successfully uses pressure differences across trapping holes and fluidic ports.
to capture cells on the device, allow them to grow and divide, and elute newborn cells that have entered the G1 phase of the cell cycle. On a device with 2109 holes, up to 1000 cells are eluted every 12 hours, with a purity of up to over 80% of the collected cells being in G1.

Although the device presented here is functional, a few deficiencies will need to be addressed in future versions of the microfluidic "baby machine." First, it was difficult to confirm the clearing of all the trapping holes while cleaning the device before reuse. Obstructed holes may hinder proper loading of cells and lower the overall yield of the device, although not necessarily altering its synchronizing functionality. Second, even after lengthy device preparation times, it was difficult to ensure the removal of all air bubbles beneath the trapping holes. Over long runs, these bubbles can expand and dislodge loaded cells. Third, beyond 36 hours of operation, aggregates of cells can form in the trapping chamber and occasionally detach from the surface, releasing non-newborn cells from the device. In spite of this, the current 36-hour window of operation may be sufficient for many biological experiments, which require only a single synchronized batch of cells and not a continuous supply.

The current yield of the synchronization device is sufficient as a demonstration and possibly for transcriptomic analysis, but would need to be greatly increased for investigations on the level of protein expression. To this end, it should be feasible to stack these 2-dimensional devices into a 3-dimensional array of trapping holes, thereby vastly increasing the yield of the device. Hence, there remains further development to improve the operation and increase the yield of the microfluidic "baby machine." As a demonstrated concept, however, since the presented technique avoids damaging chemicals, selects for age rather than size, and does not depend on surface properties of cells, it may prove to be a less perturbing and more versatile way to synchronize cells.
Appendix

A.1 Further characterizations of SMR constriction measurements

A.1.1 Supplementary figures for chapter 1

Fig. A-1. Effects of latrunculin B and nocodazole on MEF cells
Effects of latrunculin B (LatB) (5 µg/mL, 30 min) and nocodazole (Noc) (1 µg/mL, 30 min) were tested with MEF cells and compared with untreated control. (A) Treating with LatB (red bars) and Noc (green bars) both resulted in a relatively larger increase in the entry velocity than the transit velocity but LatB induced greater change than Noc. Error bars represent 95% confidence intervals. (B) LatB and Noc both induced a decrease in the passage time but the extent of the change was greater with LatB (untreated, blue, n = 570; LatB, red, n = 494; Noc, green, n = 534). Measurements were acquired with a PEG-coated channel surface and using a pressure drop of 1.35 psi.

* Data here were acquired by Dr. Sangwon Byun. These figures were taken from a manuscript that has been published: Byun, S., Son, S., Amodei, D., Cermak, N., Shaw, J. et al. “Characterizing deformability and surface friction of cancer cells.” PNAS. 2013. 110(19):7580-5. Author contributions are listed in the publication. Figure numbers have been altered to match the text of this thesis.
Fig. A-2. Effect of PEG versus PLL surfaces tested with various cell lines.

Effect of PEG versus PLL surfaces was tested with various cell lines: TMet (A and B; n = 396 for PLL, n = 572 for PEG), TnonMet (C and D; n = 220 for PLL, n = 320 for PEG), and HCC827 (E and F; n = 403 for PLL, n = 488 for PEG). For all three cell lines, PLL-coated microchannel induced a greater change in the transit velocity than the entry velocity (A, C, and E) as well as an increase in the passage time (B, D, and F). The distinct changes in entry and transit velocities caused by PEG versus PLL surfaces were consistent with those observed with H1975 cells (Fig. 1-6). Error bars represent 95% confidence intervals. Measurements were acquired using a pressure drop of 0.9 psi for the mouse cell lines (TMet, TnonMet) and 1.8 psi for the human cell line (HCC827).
A.1.2 Effects of applied pressure on passage time of cells

The pressure applied across the SMR device was varied to observe the changes in peak rise times and passage times (Fig. A-3). Peak rise times correspond to the amount of time the cell takes to traverse from the base of the cantilever to the tip of the cantilever without a constriction. This time should correspond to the linear velocity of the cells, and should thus vary proportionally with the flow rate. For all cells, the peak rise time varies linearly with the amount of applied pressure (Fig. A-3A). In contrast, the passage time may or may not vary linearly with the applied pressure (Fig. A-3B). For smaller cells, which are more similar to the size of the constriction, the passage times vary linearly; however, the larger cells seem to deviate from this pattern (e.g. cells that are 100 pg can pass through the constriction in ~0.002 s at 1.5 psi, but take ~0.2 s at 0.15 psi). This effect may be due to the shear-thinning properties of the cells [159].

Fig. A-3. Pressure variation effects on passage time and peak rise time of L1210 cells
A) The peak rise time vs. buoyant mass of L1210 cells having different applied pressures in the SMR device. Peak rise times correlate to the velocity of fluid flow in the cantilever. B) Passage time vs. buoyant mass of L1210 cells for the same measurements as in (A).
A.1.3 Effects of varying constriction dimensions

Passage time versus buoyant mass characteristics were compared for cells measured in a 4 \( \mu m \) x 100 \( \mu m \) constriction and those measured in a 6 \( \mu m \) x 50 \( \mu m \) constriction (Fig. A-4). Both constrictions were 15 \( \mu m \) deep. The cell lines measured include L1210 cells, a murine lymphoblast cell line grown in suspension, and T\textsubscript{Met} cells, a murine lung cancer line grown in adherent monolayer culture.

<table>
<thead>
<tr>
<th>4 ( \mu m ) (W) x 100 ( \mu m ) (L) Constriction</th>
<th>6 ( \mu m ) (W) x 50 ( \mu m ) (L) Constriction*</th>
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</thead>
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<tr>
<td><img src="image1.png" alt="Graph A" /></td>
<td><img src="image2.png" alt="Graph B" /></td>
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<tr>
<td><img src="image3.png" alt="Graph C" /></td>
<td><img src="image4.png" alt="Graph D" /></td>
</tr>
</tbody>
</table>

Fig. A-4. Effects of varying the constriction dimensions with different applied pressures
A) L1210 and T\textsubscript{Met} cells measured in a 4 \( \mu m \) x 100 \( \mu m \) constriction at a flow rate of 15 \( \mu L/hr \). B) L1210 and T\textsubscript{Met} cells measured in a 6 \( \mu m \) x 50 \( \mu m \) constriction at a flow rate of 15 \( \mu L/hr \). C) L1210 and T\textsubscript{Met} cells measured in a 4 \( \mu m \) x 100 \( \mu m \) constriction at a flow rate of 40 \( \mu L/hr \). D) L1210 and T\textsubscript{Met} cells measured in a 6 \( \mu m \) x 50 \( \mu m \) constriction at a flow rate of 40 \( \mu L/hr \). *Note: The 6 \( \mu m \) constriction was found to be eroding, so the width is likely to have been larger than 6 \( \mu m \).
A few results suggest potential for future studies. First, as expected, the distinction between \( T_{\text{Met}} \) versus \( \text{L1210} \) cells was maintained at a higher volumetric flow rate for the smaller constriction width. This indicates that epithelial tumor cells may be able to be distinguished from blood cells with a higher throughput in a 4 \( \mu \)m constriction device than in a 6 \( \mu \)m constriction device. Interestingly, at the lower flow rate and with a 4 \( \mu \)m constriction, the \( \text{L1210} \) cells show a kink in the passage time versus buoyant mass trend (Fig. A-4A), similar to what is seen in the \( T_{\text{Met}} \) cells in the 6 \( \mu \)m constriction. The location of the kink likely depends on the ratio of the cell diameter to the constriction width. Furthermore, using the 4 \( \mu \)m-wide constriction at a slower flow rate (Fig. A-4A), the \( \text{L1210} \) cells larger than \(~50 \) pg seem to have the same passage time versus buoyant mass characteristic as the \( T_{\text{Met}} \) cells. This is extremely fascinating, as it may suggest that under certain shear stress conditions, epithelial cells and blood cells have the same mechanical properties in spite of their diverse origins. To be conclusive, this would need to be further investigated.

A.2 SMR platform development and variations for different types of on-chip imaging and measurement

A.2.1 Combined imaging and optical lever system

During the time I began to work with the SMR, most members of the lab were using an optical lever readout system that supplied a laser beam to the underside of the cantilever, while using a separate optical system (a purchased microscope) to image the SMR from its topside. Although at the time, many had spoken of the possibility of combining the optics for both the optical lever and microscope in order to simplify the system, I was one of the first in the lab (working with Nate Cermak) to implement and build a system in this fashion in 2011 (Fig. A-5). This system is much more cost effective than purchasing a microscope. However, because the objective is shared between the read-out system and the imaging, it is difficult to use if the study being undertaken requires particular imaging specifications. For example, when I needed to detect a fluorescence signal from a cell passing through the cantilever and wanted to use a higher magnification, or wanted to image the cell in a different part of the SMR channel, it was difficult to implement. For applications requiring dynamic or altered imaging, it proved more feasible to use the purchased microscope system separate from the optical lever-read out system, described in more detail in the subsequent section.
Fig. A-5. SMR system with imaging and optical lever through same objective
A) A schematic diagram of a system combining imaging of the SMR with optical lever read out through the same objective. B) A photograph of the actual built system.
A.2.2 Addition of PMT sensor for fluorescence detection

Based on systems built by previous members of the lab, such as Sungmin Son and Amneet Gulati [122], I added a PMT sensor to a microscope to detect fluorescence signals from cells passing through the SMR (Fig. A-6). The optical lever read-out system was applied through the underside of the cantilever, based on a system built by Wesley Weng, a former graduate student in the lab. The use of the filter turret as well as being able to place the objective at the bypass channel of the SMR (which has optical access through glass instead of silicon) proved to be effective in simultaneously detecting single-cell fluorescence signal along with buoyant mass and passage time characterizations. However, the PMT setup was not sensitive enough to detect some internal fluorescence reporters, such as the YFP expressed by the Snail promoter in EpCAM\textsuperscript{ha}YFP\textsuperscript{lo} MMTV-PyMT cells (§2.2).

![Diagram of PMT setup and data visualization](image_url)

**Fig. A-6. PMT addition for fluorescence read-out**

A) Schematic diagram of PMT setup. B) Example of CMFDA-dyed pb1.3G (murine mammary carcinoma line from the Weinberg lab) mixed into mouse blood. The black circles correspond to cells that had no fluorescence signal detected by the PMT.
A.2.3 Variations of SMR constriction designs

In addition to varying on-chip imaging methods, the device design itself was also varied (Table A-1). Further characterizations of Gen4-311 and Gen5-513 are in §A.1.3, while an example peak shape from Gen4-312 is shown in Fig. A-7.

Table A-1. SMR constriction device designs*

<table>
<thead>
<tr>
<th>Device Name</th>
<th>Width of 1st Constrict. (µm)</th>
<th>Length of 1st Constrict. (µm)</th>
<th>Width of 2nd Constrict. (µm)</th>
<th>Length of 2nd Constrict. (µm)</th>
<th>Cantilever length (µm)</th>
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<td>N/A</td>
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<td>6</td>
<td>150</td>
<td>315</td>
<td></td>
</tr>
<tr>
<td>Gen4-313</td>
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<td>50</td>
<td>8</td>
<td>50</td>
<td>315</td>
<td></td>
</tr>
<tr>
<td>Gen4-314</td>
<td>6</td>
<td>50</td>
<td>7</td>
<td>50</td>
<td>315</td>
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<tr>
<td>Gen4-315</td>
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<td>150</td>
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<td>N/A</td>
<td>315</td>
<td></td>
</tr>
<tr>
<td>Gen4-316</td>
<td>6</td>
<td>50</td>
<td>6 (optical access)</td>
<td>50 (optical access)</td>
<td>315</td>
<td>Optical access not in cantilever</td>
</tr>
<tr>
<td>Gen5-510</td>
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<td>50</td>
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<td>N/A</td>
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<tr>
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<td>100</td>
<td>N/A</td>
<td>N/A</td>
<td>350</td>
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</table>

*All entrance angles are 45 degrees, and all buried channel depths are 15µm.
Fig. A-7. Example of peak shape from device Gen4-312
A screen capture of the SMR signal of a T_{Met} cell passing through a double constriction device (Gen4-312).

In addition, glass and silicon channels were fabricated such that the cells squeezing through the constrictions could be better visualized. Parallel channels with 4μm and 6μm constrictions as well as 3μm and 2μm constrictions were made. An example layout is shown in Fig. A-8.

Fig. A-8. CAD layout of alternating 4μm and 6μm constrictions
A drawing showing the layout of a silicon device with a glass layer on top, such that there is optical access to all channels. This device is composed of parallel channels, with alternating constriction widths of 4μm and 6μm. The branching of the channels is designed such that the pressure across each constriction should be the same.
A.3 Preliminary study of small cell lung cancer*

Small cell lung cancer (SCLC) comprises 10-15% of newly diagnosed lung cancers. Although the percentage appears to be small, SCLC is the deadliest and most aggressive type of lung cancer. It has been found that SCLC tumors are often composed of phenotypically different cells with neuroendocrine (NE) or mesenchymal (nonneuroendocrine, nonNE) markers, which, when propagated in vitro, can grow in a suspended or adherent state, respectively [160]. A subcutaneous injection of a mixture of NE and nonNE cells render the NE more highly metastatic than if either population were injected into the mouse alone [160].

* This study was done in collaboration with Thales Papagiannakopoulos and Sheng Rong Ng, who provided mouse blood and cell lines from the Jacks laboratory.

Fig. A-9. Passage time versus buoyant mass for SCLC cell lines and mouse CTCs
A) Passage time versus buoyant mass characteristics of 984LN nonadherent and adherent cell lines. B) Tumor cells obtained from the blood of a mouse that developed SCLC autochonously, ~12 months post infection, and had large tumors by micro-CT. C) An overlay of (A) and (B), demonstrating the similarity in mechanical properties between the 984LN nonadherent cell line and the mouse CTCs.
Fig. A-9 shows the passage time and buoyant mass properties of the nonadherent and adherent subpopulations of the 984LN SCLC cell line from the laboratory of Professor Tyler Jacks. In comparison, I have also measured the circulating tumor cells (CTCs) from a SCLC mouse model, which developed tumors autochthonously based on the deletion of p53 and Rb. Interestingly, the SCLC tumor cells found in the mouse (expressing tdTomato, sorted by FACS after red blood cell lysis) had similar mechanical properties to the nonadherent 984LN cell line. These results provide more evidence that the nonadherent subpopulation in SCLC may be the more invasive, malignant cell type, seeding new tumors at metastatic sites.

A.4 Further studies on tumor cells in circulation

As a complement to the study in §3.3, we designed a study to determine whether tumor cells that were filtered out of circulation were less deformable than cells that persisted in circulation. In §3.3, the cells in circulation may have intravasated from tumors in the lungs, but it is not known how long they have been in circulation. Thus, we injected tumor cells via the tail vein of an immunocompromised mouse to see which cells remained in circulation after two-three minutes. Fig. A-10 shows results from an experiment where we injected two doses of one million pB1.3G cells (a murine tumor cell line, dyed with CMFDA) separated by five minutes. After two to three minutes after the second dose of cells, blood was harvested from the left ventricle of the mouse. Red blood cell (RBC) lysis was performed on the blood, and the remainder of the sample was measured in the SMR with simultaneous PMT detection for the fluorescence signal of the cells.

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*This study was done in collaboration with Dr. Xin Ye of the Weinberg laboratory.*
Fig. A-11. Entry and transit velocities versus buoyant mass of tumor cells circulating in blood
A) Entry velocity versus buoyant mass of tumor cells found in the blood compared with the initially injected tumor cell line. B) Transit velocity versus buoyant mass of the same cells as in (A). The data for both panels are from the same data set as in Fig. A-10.

Interestingly, the results demonstrate that even after two to three minutes after injection through the tail vein, and passing through the lungs, cells that have long passage times are still able to persist in circulation. It seems as though any cell from the original pB1.3G population, based on its passage time alone, could have persisted in circulation without being filtered out by capillaries. Also, the trends of the entry and transit velocities of the cells persisting in circulation (Fig. A-11) do not seem to differ from the originally injected cells.

However, a few times of repeating this experiment showed that the number of cells that persist in circulation after a few minutes post-injection varies widely from mouse to mouse. A few times, there were not enough tumor cells found in the blood to proceed with an SMR measurement. Further studies would need to be carried out to determine whether a larger number of cells come back into circulation after a longer amount of time. It would be ideal to be able to carry out experiments at different time points post-injection to see if the stiffer cells preferentially get filtered out by the circulatory system over time or whether the filtration is an entirely stochastic event.
A.5 Agarose bead measurement for potential stiffness standardization*

In search for a way to standardize SMR measurements and compare the passage time characteristics to other known mechanical parameters, such as a Young’s modulus, measurements of agarose beads were performed. Beads of varying agarose (types IX and IXA) concentrations were created in the Di Carlo laboratory at the University of California, Los Angeles. They were first introduced in a glass/silicon parallel constriction device for initial imaging. Only the highest concentration of agarose (2%) had enough contrast for bright-field imaging. The bead sizes were relatively disperse, with some larger ones having diameters of ~30 µm. After attempting to apply pressure to cause the beads to pass through 6µm wide constrictions, some beads mechanically tore or broke (Fig. A-12).

**Fig. A-12.** Bright-field image of broken agarose bead

Measurements of beads containing lower concentrations of agarose were then measured in an SMR, having a 6µm-wide constriction (Fig. A-13). Due to the low density of the porous particles, their buoyant masses were lower than typical cells. However, the volume of the beads were difficult to determine since the coulter counter also could not accurately assess their volume, likely due to their porous nature. The 0.2% agarose beads, which the UCLA group deemed to be most similar to typical cells by their deformability measurement device, have a similar range of passage times as adherent cells (4T1). Their response to different shear rates also seems similar for 0.9 and 1.5 psi applied pressure (Fig. A-13E, F). A volumetric comparison, however, would be more informative than a buoyant mass comparison. Unfortunately, the study was never completed, and the information regarding the Young’s modulus of the beads (determined by AFM, for example) was never obtained. However, this study demonstrates the potential and feasibility of measuring low concentration (less than 2%) agarose as a stiffness standard in the future.

*This study was performed in collaboration with Mahdokht Masaeli from the Di Carlo laboratory at the University of California, Los Angeles.
Fig. A-13. SMR passage time versus buoyant mass measurements of agarose beads
A) Passage times of Agarose IX beads of varying agarose concentrations, plotted with L1210 (suspension) and 4T1 (adherent) cells for comparison. B) Passage times of Agarose IXA beads of varying agarose concentrations, plotted with L1210 and 4T1 cells for comparison. C) 0.2% Agarose IX and IXA beads in comparison with each other. D) 0.5% Agarose IX and IXA beads in comparison with each other. E) 0.2% Agarose IX beads measured with different applied pressures, plotted with L1210 and 4T1 cells for comparison. F) 0.2% Agarose IXA beads measured with different applied pressures, plotted with L1210 and 4T1 cells for comparison.
A.6  Further surface characterizations of cells*

The glycocalyx of cells were altered in order to characterize the effect of cell surface treatments on passage time measurements in the SMR. A list of experiments is shown in Table A-2.

First, untreated and heparinase treated (30 min in PBS) T\text{Met} cells were compared in a PEG-coated SMR, with an applied pressure of 0.9 psi. The two conditions showed similar passage times as well as entry and transit velocities, suggesting that heparinase did not induce a significant change (Fig. A-14). Interestingly, a significant second population of cells or debris was detected. The whole population can be divided into two distinct groups, following the green dotted line (Fig. A-14B). This can be similarly observed after cells undergo freeze-thaw cycle, which creates potential debris (lysed cells) with different passage time characteristics. After 30 min treatment with PBS (with or without heparinase), spontaneous cell dissociation from the surface and ~10-30% cell death were observed (Nathan Stebbins). However, it seems unlikely that the PBS induced a large amount of cell death and debris. Moreover, the number of small cells is much greater than 10-30% of the total number of cells counted during the measurement. It is possible that this population represents normal but small cells. One caveat is that the changes described above after incubation in PBS may have induced a change in cell deformability and masked the effect of heparinase.

As a follow-up experiment, T\text{Met} cells were treated with heparinase in DMEM culture medium and measured in a PLL-coated SMR (Fig. A-15). Cells were detached from the surface using Non-enzymatic Cell Dissociation Solution in PBS (Sigma). The same applied pressure of 0.9 psi was used. Again, no significant change in passage time was observed. The protocol for non-enzymatic dissociation is as follows. 1) All materials are warmed to 37°C before use. 2) Wash cells once with PBS. 3) Incubate cells with a few mL of the dissociation solution for 5 min in the 37°C incubator. 4) Add an equal volume of PBS and dislodge the remaining cells with a pipette. 5) Pellet cells. 6) Re-suspend in media.

Next, to test the effect the duration of trypLE (cell dissociation) treatment on passage time, Lewis Lung Cancer (LLC) cells were treated with trypLE for 5 min (short) and 20 min (long) for the detachment and measured in a PLL-coated SMR. Opti-MEM was used for culture medium. The same applied pressure of 0.9 psi was used. As a result, longer incubation in trypLE resulted in slightly shorter passage times (Fig. A-16A). The transit velocity, but not the entry velocity, was affected by longer treatment (Fig. A-16B, C). The transit vs. entry velocities plot supports the fact that the change was surface-dominant (Fig. A-16D). Also, by the microscope, it was readily observed that cells with the shorter treatment were much stickier than those with the longer treatment.

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* This study was mostly carried out by Dr. Sangwon Byun from the Manalis Laboratory in collaboration with Nathan Stebbins from the Sasisekharan Laboratory. I also measured a subset of the cells in this study simultaneously with Dr. Byun and performed some analysis on these replicate experiments, although they are not shown due to redundancy. The results shown here were prepared and written by Dr. Byun, and edited by me for completeness of the record of the Manalis Lab.
Table A-2. List of experiments

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<th>Surface</th>
<th>Conditions</th>
<th>Applied Pressure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tmet</td>
<td>PEG</td>
<td>Control (30 min, PBS)</td>
<td>0.9 psi</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heparinase treated (30 min, PBS)</td>
<td></td>
</tr>
<tr>
<td>Tmet</td>
<td>PLL</td>
<td>Control (30 min, DMEM)</td>
<td>0.9 psi</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heparinase treated (30 min, DMEM)</td>
<td></td>
</tr>
<tr>
<td>LLC</td>
<td>PLL</td>
<td>TrypLE treatment for 2 min</td>
<td>0.9 psi</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TrypLE treatment for 20 min</td>
<td></td>
</tr>
<tr>
<td>LLC</td>
<td>PLL</td>
<td>Control (30 min in PBS)</td>
<td>0.9 psi</td>
</tr>
<tr>
<td></td>
<td></td>
<td>400 nM heparinase I + 400 nM heparinase III (30 min, PBS)</td>
<td></td>
</tr>
<tr>
<td>LLC</td>
<td>PEG</td>
<td>TrypLE treatment for 2 min</td>
<td>0.9 psi</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Incubation in PBS for 30 min</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Incubation in OptiMEM for 30 min</td>
<td></td>
</tr>
<tr>
<td>LLC</td>
<td>PLL</td>
<td>Control (OptiMEM)</td>
<td>0.9 psi</td>
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<tr>
<td></td>
<td></td>
<td>Sialidase treated</td>
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Fig A-14. Heparinase-treated and untreated T_Met cells in PBS
A) Passage time vs. buoyant mass of heparinase-treated and untreated (control) T_Met cells in PBS. B) A second population of cells or debris is apparent after incubation in PBS. C) Entry velocity vs. buoyant mass for the same data set as in (A). D) Transit velocity vs. buoyant mass for the same data set.
Fig A-15. Passage time of heparinase-treated and untreated T_{Met} cells in DMEM medium

Fig A-16. LLC cells having been in trypLE for different amounts of time
A) Passage time vs. buoyant mass of LLC cells, having been in trypLE for various amounts of time (short: 5 min, long: 20 min). B) Entry velocity vs. buoyant mass for the same data set. C) Transit velocity vs. buoyant mass. D) Transit velocity vs. entry velocity
Then, the combined effect of heparinase I and III was tested on LLC cells. Untreated LLC cells (30 min in PBS) were compared to heparinase-treated cells (30 min in 400 nM heparinase I + 400 nM heparinase III in PBS). No significant change in passage time was detected (Fig. A-17). However, distinct subpopulations were observed with both conditions, which was not observed in the previous experiments (Fig. A-16) that compared short and long trypLE treatment in Opti-MEM (Opti-MEM is a medium with minimal amounts of protein). The subpopulation having a smaller size showed longer passage times, which was similar to the result from T_{Met} cells (Fig. A-14), where it seemed that incubating cells in PBS might cause changes in cells that were distinguishable by the SMR but not by trypan-blue.

In order to test whether the subpopulation described above was a result from harsh treatment, i.e., 30 min incubation in PBS, LLC cells were treated under three different conditions: incubated with trypsin for 2 min, in PBS for 30 min, or in culture medium for 30 min before measurement. As a note, LLC cells can be collected without trypsin, so incubating in trypsin was considered as a surface treatment. Also, the SMR surface was coated with PEG for these measurements. In all three conditions of treatment, a subpopulation was observed (Fig. A-18). The relative count of cells or debris within the subpopulation was highest with PBS and lowest with trypsin (Fig. A-18B). Interestingly, the 2 min trypsin treatment resulted in a slightly faster passage time but it is not clear whether such a short treatment was enough to change the transit through the PEG-coated surface (Fig. A-18D, E). Also, the SMR peaks from the subpopulation were compared to those from the main population (Fig. A-19). The cells from the main population showed typical, canonical peak shapes as seen in most cell lines (Fig. A-19B). However, some cells from the subpopulation showed different shapes (Fig. A-19C, especially top row), which exhibited a more dramatic transit through the constriction. Those cells were much smaller than the main population, suggesting that they might represent only a fragment of a cell.

Finally, the effect of sialidase on the passage time of LLC cells was examined. Untreated (in culture medium) and sialidase-treated LLC cells were measure in a PLL-coated SMR (Fig. A-20). Sialidase treatment reduced cell viability, so for future studies, the treatment needs to be modified. Sialidase treatment increased the passage time, which was counter-intuitive, and this result might be related to the cell death caused by the treatment. Separation between the main population and subpopulation became unclear after sialidase treatment. Interestingly, the change in velocity was surface-dominant since the relative change in transit velocity was greater than that in entry velocity (Figs. A-20B, C). SMR peak shapes were compared within the two populations (Figs. A-21). The cells from main population showed canonical peak shapes even with sialidase treatment (Fig. A-21B, left side). However, most of cells from the smaller, subpopulation did not show canonical peak shapes. Those SMR peaks were also different from the peaks with dramatic change observed with the previous experiment (Fig. A-19). Further study is needed to confirm whether the cell passage changes seen with sialidase-treatment were induced by cell death.
Fig. A-17. Untreated (control) and heparinase-treated LLC cells
A) Passage time vs. buoyant mass of untreated and heparinase-treated LLC cells. B) Entry velocity vs. buoyant mass. C) Transit velocity vs. buoyant mass. D) Transit velocity vs. entry velocity.
Fig. A-18. LLC cells treated with trypsin, PBS, or OptiMEM
LLC cells were incubated in trypsin for 2 min (red), or PBS for 30 min (blue), or OptiMEM for 30 min (green) prior to SMR measurement. A) Passage time vs. buoyant mass. B) Counts of cells based on buoyant mass. C) Entry velocity vs. buoyant mass. D) Transit velocity vs. buoyant mass. E) Transit velocity vs. entry velocity.

Fig. A-19. Comparison of SMR peak shapes between cells in different subpopulations
A) For the same data in Fig. A-18, SMR measurements were compared for cells having passage times between 0.1 s and 0.5 s. B) For cells having a buoyant mass between 140 pg and 150 pg, the frequency vs. time SMR readout had canonical peak shapes, as found with most cell lines. C) For cells in the smaller subpopulation, having a buoyant mass less than 60 pg, the peak shapes demonstrated varied patterns both in the entry and transit regions as the cells passed through the constriction.
Fig. A-20. Untreated and sialidase-treated LLC cells
A) Passage time vs. buoyant mass of untreated (control) and sialidase-treated LLC cells. B) Entry velocity vs. buoyant mass. C) Transit velocity vs. buoyant mass. D) Transit velocity vs. entry velocity.
Fig. A-21. SMR peak shapes for untreated and sialidase-treated LLC cells within different subpopulations
A) Representative frequency vs. time traces of untreated (control) LLC cells as they pass through the SMR. B) Representative frequency vs. time traces of sialidase-treated LLC cells as they pass through the SMR. Cells shown have a passage time between 0.07 s and 0.1 s. The left column for both panels are cells from the main population of cells, having a buoyant mass between 100 pg and 125 pg; whereas the right column for both panels are cells from the subpopulation of cells having a buoyant mass less than 70 pg.
A.7 Characterizing heterogeneity in single-cell biophysical properties using SMR passage time measurements*

Mechanical properties of cells are largely governed by cytoskeletal proteins, such as actin filaments, microtubules, and nuclear lamina. Disrupting such cytoskeletal structures may lead to a change in holistic cellular biophysical properties, such as the viscoelasticity of individual cells. Although biophysical parameters of cells undergoing cytoskeletal changes have been studied in great detail, how those parameters become more homogenous or heterogeneous are not well understood. Here we use drug treatments and genetically altered cells to study the effects of cytoskeletal changes on heterogeneity in biophysical properties by measuring passage time using an SMR with a 6μm-wide constriction. Lamin A/C deficiency in mouse embryonic fibroblasts (Lmna +/- MEF), which is known to reduce cell stiffness while the cells are in an adherent state, significantly decreases the passage time through the SMR constriction. We also find that disrupting actin filaments by latrunculin B (LatB) or microtubules by nocodazole (Noc) both induce a decrease in passage time in Lmna +/- MEFs. These results, however, are unexpected since the mechanical properties of Lmna +/- MEFs measured in an adherent state (by ballistic intracellular nanorheology) are not affected by the same cytoskeletal disruption. Despite these significant effects on passage time, lack of lamin A/C and drug treatments do not affect variability in passage time. In contrast, treating FL5.12 cells with LatB, which naturally grow in suspension, induces a decrease in passage time and also a decrease in heterogeneity of passage times, whereas treating with Noc affect neither. Changes in passage time induced by LatB and Noc together are similar to those by LatB alone. High throughput cellular phenotyping (HTPC) on FL5.12 cells shows that the heterogeneity in microtubules is decreased by all three treatments. However, the heterogeneity in actin filaments is decreased by LatB and Noc separately but not by LatB and Noc together, suggesting an interplay between actin and microtubules. These results suggest that cytoskeletal disruption can attribute to the heterogeneity in passage time, but that its effect is limited by anchorage dependence.

* This study was mostly carried out by Dr. Sangwon Byun from the Manalis Laboratory in collaboration with the Wirtz Laboratory (especially Dr. Shyam Khatau and Jorge Marchand) at Johns Hopkins University. I also measured the FL5.12 cells in this study simultaneously with Dr. Byun and performed some analysis on these replicate experiments, although they are not shown due to redundancy. The results were prepared and written by Dr. Byun, and edited by me for completeness of the record of the Manalis Lab.
Fig. A-22. SMR measurements of wild-type and lamin A/C knock-out MEFs
Passage time measurement shows that MEFs without lamin A/C (Lmna -/-) are more deformable than the wild-type (Lmna +/-). The change in deformability (seen in entry velocity) is a more dominant factor causing a decrease in passage time than friction (seen in transit velocity).

Fig. A-23. Offset in passage time for MEF cells after treatment with LatB or Noc
Disrupting the actin filaments by latrunculin B (LatB) or the microtubules by nocodazole (Noc) both induce a decrease in passage time in Lmna +/- and Lmna -/- MEFs. These results are unexpected since mechanical properties of Lmna -/- MEFs measured in adherent state are not affected by the same cytoskeletal disruption.
Fig. A-24. Passage times of Lmna +/+ MEFs treated with LatB compared with those of Lmna-/- MEFs. The passage times of wild-type (Lmna +/+) MEFs that have been treated with LatB are similar to those of Lmna-/- MEFs without any treatment. This is consistent with a previous study by nanorheology.

Fig. A-25. Trajectories of SMR peaks reveal cells with a distinct ("bumpy") peak pattern. After LatB treatment, certain cells demonstrate a bump in the transition region of the SMR peak shape, while entering and transiting through the constriction.
Fig. A-26. More “bumpy” peaks are observed with Lmna +/+ than Lmna -/- MEFs after LatB treatment.

Fig. A-27. Heterogeneity in passage times
The coefficient of variation (CV) in the passage times of MEFs is not affected by lamin A/C deficiency or drug treatments. The CV of L1210s (or FL5s), however, is significantly lower than that of MEFs. Analysis was performed by Nathan Cermak.

Fig. A-28. Treating FL5.12 cells with LatB induces a decrease in passage time and heterogeneity. LatB treatment of FL5.12 cells causes a decrease in both passage time and the heterogeneity of passage times; whereas Noc treatment affects neither. Changes in passage time induced by LatB and Noc together are similar to those by LatB alone.
Fig. A-29. High throughput cellular phenotyping (HTPC) on FL5.12 cells
HTPC shows that heterogeneity in microtubules is decreased by all three treatments: LatB alone, Noc alone, and LatB and Noc together. However, heterogeneity in actin filaments is decreased by LatB and Noc separately, but not by LatB and Noc together, suggesting interplay between actin and microtubules (Jorge Marchand, JHU).
A.8 Characterizing the effect of Mena and Mena\textsuperscript{INV} on the stiffness of cancer cells\textsuperscript{*}

All measurements were performed by Dr. Sangwon Byun of the Manalis Lab, and all cell lines (MDA-MB-231) were provided by Dr. M. J. Oudin of the Gertler group. The applied pressure for all measurements was 0.9 psi.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{passage_time_vs_buoyant_mass.png}
\caption{Passage time vs. buoyant mass of control, Mena, and Mena\textsuperscript{INV} cells. For large cells, Mena\textsuperscript{INV} cells had the longest passage times. However, more large cells from the Mena\textsuperscript{INV} cell line were counted than for Mena or control cells, since Mena\textsuperscript{INV} cells were on average larger in volume. It is undetermined whether the longer passage times for Mena\textsuperscript{INV} cells are due to volume alone, or whether they are indeed stiffer than control cells of similar size.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{volumes.png}
\caption{Volumes of control, Mena, and Mena\textsuperscript{INV} cells measured by a Coulter counter.}
\end{figure}

\textsuperscript{*} This study was carried out entirely by Dr. Sangwon Byun from the Manalis Laboratory in collaboration with the Dr. Madeleine Oudin from the Gertler Laboratory. The results were prepared and written by Dr. Byun, and included here for the completeness of the record for the Manalis Lab.
Fig. A-32. Entry and transit velocities of control, Mena, and Mena^{INV} cells
Entry and transit velocity trends showed similar results as the passage time.

Fig. A-33. Passage time vs. volume instead of buoyant mass.
The buoyant mass of each cell was converted to the volume using the density estimated from the population average of buoyant mass (SMR) and the volume (Coulter counter) of each cell line (Control = 1.0521 g/ml, Mena = 1.0547 g/ml, Mena^{INV} = 1.0535 g/ml). Since densities were similar to each other, conversion didn't cause a significant shift in passage time. However, with the volume, Mena^{INV} and control cells became slightly more separated.
Fig. A-34. Passage time plotted vs. binned volumes and buoyant masses

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Bibliography


