PHYSICAL MECHANISMS AND HIGH-THROUGHPUT IMAGING OF TUMOR CELL MIGRATION IN CONFINING MICROENVIRONMENTS

by

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Metastasis, the process by which cancer cells spread from a primary tumor to other parts of the body, is responsible for ~90% of cancer-related deaths. Migration of cancer cells away from a primary tumor is crucial in the metastatic cascade. Advances in intravital imaging have elucidated the importance of cancer cell migration through confining tunnel-like tracks in the tumor microenvironment. Confined migration can occur by a variety of mechanisms, confounding efforts to abate metastasis.

Following a discussion of bioengineering tools used in confined migration studies and mechanisms that use of these tools have revealed, we describe and characterize a high-throughput lens-free imaging (LFI) system for use in cell migration assays. The LFI system offers the temporal and spatial resolution needed for time lapse analysis of cell migration without requiring specialized equipment to support cell survival on microscope stages. Instead, the compact size of the LFI system enables these experiments to be performed in a traditional cell culture incubator. After thoroughly benchmarking LFI results against live-cell phase contrast microscopy for random, microcontact printing, and microchannel motility assays, we demonstrate how the migration of MDA-MB-231 and MCF7 breast adenocarcinoma cells becomes increasingly efficient as cells are confined first to microcontact printed lines and then within three-dimensional (3D) microchannels.

We next use microfabrication techniques to probe how distinct physical microenvironments modulate the decision making of MDA-MB-231 and HT1080 cells. The peritumoral physical microenvironment consists of complex topologies that influence cell migration, but the decision making of cells upon encountering anisotropic
physical cues has yet to be elucidated. Lateral two-dimensional (2D) confinement on printed lines and full cell confinement in narrow 3D microchannels led MDA-MB-231 cells to preferentially enter wider regions at asymmetric bifurcations. In contrast, when microchannels wider than the cell body were used, cells elongated along one side wall and were contact guided to a contiguous branch channel independently of branch channel width. Inhibition of contractility in both MDA-MB-231 and HT1080 cells decreased contact guidance along a topographical feature, while inhibition of Cdc42 promoted it. Overall, our results elucidate the critical role of the physical microenvironment in regulating cell migration.

Advisor: Dr. Konstantinos Konstantopoulos
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DEDICATION

To the women in my life who made this work possible: Anne, Annie, and Michelle.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>iv</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xiii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xiv</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>CELL MIGRATION THROUGH MICROCHANNEL-LIKE SPACES IN VIVO</td>
<td>4</td>
</tr>
<tr>
<td>2.1. Topographic Cues Potentiate Rapid Tumor Cell Migration Through</td>
<td>4</td>
</tr>
<tr>
<td>Tunnel-Like Spaces</td>
<td></td>
</tr>
<tr>
<td>BIOENGINEERING METHODS FOR STUDYING CONFINED MIGRATION</td>
<td>7</td>
</tr>
<tr>
<td>3.1. Fabrication of Microfluidic Devices for Confined Migration Studies</td>
<td>8</td>
</tr>
<tr>
<td>3.2. PDMS Microfluidic Microchannel Devices</td>
<td>10</td>
</tr>
<tr>
<td>3.2.1. Straight PDMS microchannels</td>
<td>11</td>
</tr>
<tr>
<td>3.2.2. Chemical gradients</td>
<td>12</td>
</tr>
<tr>
<td>3.2.3. Voltage gradients</td>
<td>13</td>
</tr>
<tr>
<td>3.2.4. Physical gradients and cell decision making</td>
<td>14</td>
</tr>
<tr>
<td>3.3. Grooved Substrates</td>
<td>15</td>
</tr>
<tr>
<td>3.4. Microprinted Lines and Islands</td>
<td>16</td>
</tr>
<tr>
<td>3.4.1. Microprinted 1D lines to study cell migration</td>
<td>17</td>
</tr>
<tr>
<td>3.4.2. Microcontact printed islands</td>
<td>18</td>
</tr>
<tr>
<td>3.4.3. Micropatterned stiffness islands</td>
<td>19</td>
</tr>
<tr>
<td>3.5. Vertical Confinement</td>
<td>19</td>
</tr>
<tr>
<td>3.6. Patterned Gels</td>
<td>20</td>
</tr>
<tr>
<td>3.6.1. Variable pore sizes via controlled polymerization</td>
<td>20</td>
</tr>
<tr>
<td>3.6.2. Photopatterned gels</td>
<td>21</td>
</tr>
<tr>
<td>3.6.3. Micromolded collagen and polyacrylamide hydrogels</td>
<td>22</td>
</tr>
<tr>
<td>3.7. Conclusion</td>
<td>23</td>
</tr>
</tbody>
</table>
MECHANISMS AND COMPENSATION STRATEGIES FOR CONFINED MIGRATION ................................................................................................. 24

4.1. Topographical Features Elicit Spontaneous Cytoskeletal Alignment and Persistent Cell Migration .................................................................. 24

4.2. Nuclear Confinement Modulates Tumor Cell Migration and Division ........... 27

4.3. Tumor Cells Migrate Efficiently Through Microtracks Independently of MMPs ......................................................................................... 30

4.4. The Complex Roles of Actomyosin Contractility and Adhesions in Confined Migration ............................................................................. 31

4.5. Cellular Protrusions Optimize Confined Migration ........................................ 35

4.6. Confinement Mediates Collective-to-Single Cell and Mesenchymal-to-
 Amoeboid Transitions .................................................................................. 36

4.7. Key Differences in Confined Migration Assays .............................................. 38

LENS-FREE IMAGING FOR TIME LAPSE CELL MOTILITY ASSAYS .......... 40

5.1. Introduction .................................................................................................. 40

5.2. Materials and Methods .................................................................................. 42

5.2.1. Lens free imaging (LFI) setup .................................................................. 42

5.2.2. Phase contrast microscopy setup .............................................................. 46

5.2.3. Master fabrication .................................................................................... 46

5.2.4. PDMS Micromolding ............................................................................ 47

5.2.5. Cell culture ............................................................................................. 50

5.2.6. Migration assay preparation ................................................................... 50

5.2.7. Cell tracking ........................................................................................... 54

5.2.8. Statistics ................................................................................................ 55

5.3. Results ......................................................................................................... 56

5.3.1. Compact lens-free imaging platform produces large field-of-view images in which cell morphology is clearly visible ................................. 56

5.3.2. Lens-free and phase contrast imaging produce quantitatively similar results for a wide range of single-cell migration assays ........................................... 57

5.4. Discussion .................................................................................................. 79

PHYSICAL CONFINEMENT DRIVES RAPID AND PERSISTENT MIGRATION OF MDA-MB-231 AND MCF7 CELLS ........................................ 81

6.1. Introduction .................................................................................................. 81

6.2. Materials and Methods ............................................................................... 82
6.2.1. Cell culture, imaging methods, and assay preparation ........................................ 82
6.2.2. Cell tracking ........................................................................................................ 82
6.2.3. Statistics ............................................................................................................ 83

6.3. Results .................................................................................................................. 84
6.3.1. 1D vs. 3D physical confinement differentially regulates cell motility. .............. 84
6.3.2. Microenvironmental exploration by metastatic tumor cells is aided by rapid
migration with minimal speed variance ................................................................... 91

6.4. Discussion ............................................................................................................. 97

MORPHOLOGICAL ANALYSIS OF MDA-MB-231 CELL MIGRATION IN
VARIED MICROENVIRONMENTS ............................................................................. 99

7.1. Introduction .......................................................................................................... 99

7.2. Materials and Methods ....................................................................................... 100
7.2.1. Cell lines and cell culture .................................................................................. 100
7.2.2. Fabrication of a microfluidic device for examination of cell migration in multiple
topographic regimes .............................................................................................. 101
7.2.3. Microchannel cell migration experiments ......................................................... 104
7.2.4. Microfluidic template printing experiments ....................................................... 105
7.2.5. Cell tracking .................................................................................................... 106
7.2.6. Statistics ........................................................................................................... 107

7.3. Results .................................................................................................................. 107
7.3.1. Confinement promotes persistent migration of MDA-MB-231 cells. .............. 107
7.3.2. Changes in MDA-MB-231 cell morphodynamics underlie confinement-induced
cell persistence ....................................................................................................... 112

7.4. Discussion ............................................................................................................. 120

THE INTERPLAY OF THE PHYSICAL MICROENVIRONMENT,
CONTACT GUIDANCE, AND CELL SIGNALING IN CELL DECISION
MAKING .................................................................................................................... 122

8.1. Introduction .......................................................................................................... 122

8.2. Materials and Methods ....................................................................................... 124
8.2.1. Cell lines and siRNA experiments .................................................................. 124
8.2.2. Western blot analysis ....................................................................................... 125
8.2.3. Fabrication of a microfluidic device for examination of cell migration in multiple
topographic regimes .............................................................................................. 126
8.2.4. Microchannel and microfluidic template printing cell migration experiments..... 130
8.2.5. Cell tracking ........................................................................................................ 130
8.2.6. Statistics ............................................................................................................... 131

8.3. Results .................................................................................................................... 132
8.3.1. The distinct roles of confinement and contact guidance in MDA-MB-231 cell decision making at bifurcations .............................................................. 132
8.3.2. The critical length scales affecting MDA-MB-231 cell decision making at bifurcations from partial versus full confinement ..................................................................... 138
8.3.3. The critical role of integrin-mediated adhesion in the contact guidance of MDA-MB-231 and HT1080 cells. ......................................................................................... 141
8.3.4. The differential effects of cell contractility in MDA-MB-231 cell decision making at bifurcations from partial versus full confinement ........................................... 145
8.3.5. Inhibition of Cdc42 increases contact guided-mediated decision making in MDA-MB-231 and HT1080 cells. .............................................................. 153

8.4. Discussion ............................................................................................................... 158

FUTURE DIRECTIONS AND CONCLUDING REMARKS ............................................ 163
9.1. Introduction ............................................................................................................. 163

9.2. Translational Applications of Studying Cell Behavior in Confinement .......... 163
9.2.1. Anti-metastatic effects of modulating the tumor microenvironment .................. 164
9.2.2. Elucidation of single-cell vs. population-level characteristics ......................... 165
9.2.3. High-throughput drug screening assays in physiologically-relevant microenvironments ................................................................................................................................. 166
9.2.4. Point-of-care microfluidic devices using whole blood from patients ................ 166

9.3. A Cell Decision Making Device for Assessing Metastatic Propensity ........... 168
9.3.1. Microfluidic device design for metastatic propensity assay ............................... 168
9.3.2. Characterization of migratory and non-migratory cells in human breast cancer cell lines ......................................................................................................................... 169
9.3.3. Correlation of migration in the microfluidic device with metastatic propensity ... 171
9.3.4. Isolation of migratory MDA-MB-231 cells .......................................................... 174
9.3.5. Suggestions for future experiments: Y-microchannel metastatic propensity assay ...... 176

9.4. Outlook and Perspectives: The Future of Microchannel Migration Assays. 177

BIBLIOGRAPHY ............................................................................................................. 180
CURRICULUM VITAE ..................................................................................................... 210
LIST OF TABLES

Table 4-1. Morphological characteristics of confined cells ........................................... 25

Table 9-1. Metastatic propensity of human breast cancer cell lines correlates with
the percentage of migratory cells in Y-shaped microchannels................................. 172

Table 9-2. The migration of triple negative breast cancer cells in Y-shaped
microchannels is differentially impacted by PI3K inhibition................................. 173
LIST OF FIGURES

Figure 3-1. Overview of a typical microfabrication process........................................... 9

Figure 5-1. Lens-free imaging (LFI) principle of operation, setup, and characteristics. ....................................................................................................................... 43

Figure 5-2. LFI platform setup. ......................................................................................... 45

Figure 5-3. LFI image quality is dependent on the PDMS micromolding technique used. ....................................................................................................................... 49

Figure 5-4. Representative images from the cell migration assays illustrate the large field of view of the LFI platform................................................................. 58

Figure 5-5. Phase contrast and LFI imaging platforms generate similar results for random motility assays. ................................................................. 60

Figure 5-6. Phase contrast and LFI imaging platforms generate similar results for microcontact printing migration assays. ................................................................. 62

Figure 5-7. Phase contrast and LFI imaging platforms generate similar results for microchannel migration assays......................................................... 64

Figure 5-8. Phase contrast and LFI imaging platforms generate similar results for random motility assays over longer (8 h) imaging times. ......................... 67

Figure 5-9. Phase contrast and LFI imaging platforms generate similar results for microcontact printing migration assays over longer imaging times......... 69

Figure 5-10. Phase contrast and LFI imaging platforms generate similar results for microchannel migration assays over longer imaging times.................. 71

Figure 5-11. Quantitative descriptors of random MDA-MB-231 and MCF7 cell motility are dependent on the tracking time used during the experiment. ........... 73

Figure 5-12. Velocity and persistence but not speed are dependent on the tracking time used during migration of MDA-MB-231 and MCF7 cells on microcontact printed lines. ......................................................................................... 75

Figure 5-13. Quantitative descriptors of MDA-MB-231 and MCF7 cell migration in confining microchannels display different dependence on tracking time. ........... 77

Figure 6-1. Increasing confinement results in more efficient migration of MDA-MB-231 and MCF7 cells (LFI imaging). ................................................................. 85

Figure 6-2. Increasing confinement results in more efficient migration of MDA-MB-231 and MCF7 cells (phase contrast imaging).................................................. 87
Figure 6-3. Increasing confinement results in more efficient cell migration of MDA-MB-231 and MCF7 cells, regardless of cell tracking time. 89

Figure 6-4. The fastest MDA-MB-231 cells migrate with the smallest coefficient of variation in speed, regardless of the migration microenvironment, in cells imaged with the LFI platform. 92

Figure 6-5. The fastest MDA-MB-231 cells migrate with the smallest coefficient of variation in speed, regardless of the migration microenvironment, in cells imaged with phase contrast microscopy. 93

Figure 6-6. The fastest MDA-MB-231 cells migrate with the smallest coefficient of variation in speed, regardless of the migration microenvironment, for longer cell migration periods. 94

Fig. 6-7. Faster MDA-MB-231 cells explore more of their microenvironment, with confinement enhancing displacement from initial cell position. 96

Figure 7-1. Design of microfluidic device and microcontact printed surfaces. 103

Figure 7-2. The physical microenvironment affects the persistence of MDA-MB-231 cell migration through changes in cell shape and morphodynamics. 109

Figure 7-3. Confocal reconstruction of MDA-MB-231 cell F-actin architecture in 20 µm-wide feeder microchannels. 111

Figure 7-4. Shape factors of MDA-MB-231 cells in different physical microenvironments. 115

Figure 7-5. Physical confinement reduces the extent and dynamics of protrusions and causes persistent alignment for MDA-MB-231. 117

Figure 7-6. The shape and morphodynamics of migrating MDA-MB-231 cells are modulated by the microenvironment. 118

Figure 8-1. Design of microfluidic device and microcontact printed surfaces for cell decision making studies. 128

Figure 8-2. Representative images of MDA-MB-231 cells making decisions from various feeder channel microenvironments. 133

Figure 8-3. The distinct roles of confinement and contact guidance in MDA-MB-231 cell decision making at bifurcations. 134

Figure 8-4. Contact guidance regulates decision making of MDA-MB-231 cells at bifurcations stemming from wide feeder channels. 137

Figure 8-5. The critical length scales affecting MDA-MB-231 cell decision making at bifurcations from partial versus full confinement. 139
Figure 8-6. The critical role of integrin-mediated adhesion in the contact guidance of MDA-MB-231 cells. ........................................................................................................... 143

Figure 8-7. The critical role of integrin-mediated adhesion in the contact guidance of HT1080 cells. .................................................................................................................. 144

Figure 8-8. The differential effects of cell contractility in the contact guidance and decision making of MDA-MB-231 cells at bifurcations from partial versus full confinement. ........................................................................................................ 146

Figure 8-9. The roles of actomyosin contractility, Cdc42, and Rac1 in the migration and contact guidance of HT1080 fibrosarcoma cells. ....................................................... 148

Figure 8-10. Individual knockdown of non-muscle myosin II isoforms, MIIA and MIIB, does not modulate contact guidance of MDA-MB-231 cells. ......................... 151

Figure 8-11. Inhibition of Cdc42 increases contact guidance-mediated decision making in MDA-MB-231 cells........................................................................................................ 154

Figure 8-12. The effect of inhibition of Rac1 on MDA-MB-231 cell contact guidance-mediated decision making at bifurcations. .......................................................... 157

Figure 8-13. Concurrent inhibition of both RhoA/ROCK and MLCK is required for modulation of contact guidance in MDA-MB-231 cells. ............................................. 160

Figure 9-1. Spontaneous segregation of MDA-MB-231 cells into migratory and non-migratory subpopulations in Y-shaped microchannels. ............................. 170

Figure 9-2. Isolated migratory MDA-MB-231 cells are more likely to cause micrometastases following orthotopic injection in mice................................................. 175
Chapter 1

INTRODUCTION

Cancer metastasis, the process by which tumor cells disseminate from a primary tumor to secondary tissues, is responsible for about 90% of deaths from solid tumors (Wirtz et al., 2011). The metastatic cascade is complex, encompassing migration of cells away from a primary tumor, intravasation of these cells into the circulatory or lymph systems, transit of cells through these systems, extravasation to secondary tissues, and formation of a secondary tumor (Wirtz et al., 2011; Gupta et al., 2006; Talmadge et al., 2010). Cell migration is a vital component in these metastatic processes (Talmadge et al., 2010; Wirtz et al., 2011).

Recent intravital microscopy data have shown the prominent role of migration through track-like spaces during tumor cell migration at both the primary tumor and metastatic sites. Migration tracks in vivo may be either endogeneous features of the tissues (Alexander et al., 2008; Alexander et al., 2013), or they may be created by tumor or tumor-associated cells (Friedl et al., 2009; Gaggioli, 2008; Gaggioli et al., 2007; Hanahan et al., 2012). Importantly, migration mechanisms through these environments are not predicted by two-dimensional (2D) or gel-based migration assays (Stroka et al., 2014b; Hung et al., 2013; Balzer et al., 2012; Tozluoglu et al., 2013). Engineered in vitro environments have enabled study of how physiologically-relevant confinement affects cellular processes such as migration and division. Understanding cancer cell migration
through confining tracks in the human body will be key to future treatment strategies aiming to inhibit metastasis.

In this dissertation, *in vivo* evidence of tunnel-like migration spaces is first discussed to illustrate why these tracks are important in the context of cancer cell migration (Chapter 2). Bioengineering approaches to impose confinement on cells in controlled, observable, and reproducible environments are then summarized (Chapter 3). These engineering tools have greatly increased our understanding of migration mechanisms and compensation strategies that tumor cells use to navigate the microenvironment (Chapter 4). To improve the accessibility and throughput of such tools and assays, we optimized and benchmarked a novel lens-free imaging platform for use with cell migration assays (Chapter 5).

Having established tools to study confined migration, we asked how confinement affects the characteristics and mechanisms of cell motility. We have previously reported a microfluidic device containing an array of microchannels with cross-sectional areas on the scale of pores found *in vivo* (Tong et al., 2012; Wolf et al., 2009). The channels are fabricated using photolithography and can be designed in a number of widths, heights, lengths, and geometrical designs. Use of microfabrication techniques revealed that physical confinement potentiates rapid and persistent migration of MDA-MB-231 breast adenocarcinoma cells, with three-dimensional (3D) confinement within microchannels leading to more persistent migration than one-dimensional (1D) lateral confinement on microcontact-printed lines (Chapter 6). Detailed morphometric analysis of these cells on 2D surfaces, on microcontact-printed lines, and within microchannels elucidated how confinement drives cell persistence by suppressing cell protrusions and aligning cells
along topographical features (Chapter 7). Physical confinement also mediates cell
decision making at bifurcation points in a manner dependent on the dimensionality and
length scales of the confining substrate. Furthermore, for MDA-MB-231 and HT1080
cells inhibition of actomyosin contractility decreases the tendency of cells to migrate
along a topographical feature, while inhibition of Cdc42 activity promotes such contact-
guided migration (Chapter 8).

We conclude by discussing potential translational applications of biophysical and
microfluidic microchannel assays, including our preliminary work using a microchannel
migration model to assess the metastatic propensity of different breast cancer cell lines
(Chapter 9). In sum, this dissertation reviews and illustrates current and potential uses of
confined migration assays to understand cell biology and improve human health.
Chapter 2

CELL MIGRATION THROUGH MICROCHANNEL-LIKE SPACES IN VIVO

Time lapse, deep-tissue imaging made possible by advances in intravital microscopy has illustrated the importance of tumor cell migration through confining, tunnel-like tracks in vivo. The variety of microenvironments through which cancer cells migrate in vivo (Wolf et al., 2009; Alexander et al., 2008; Alexander et al., 2013; Gritsenko et al., 2012; Schmidt et al., 2010; Weigelin et al., 2012), and the diversity of migration mechanisms available to tumor cells (Friedl et al., 2011; Schmidt et al., 2010; Wolf et al., 2011; Wolf et al., 2006), confounds efforts to abate metastasis-initiating migration in a clinical setting. Increasingly, researchers are characterizing the physical structures of the in vivo microenvironment to better understand the full regime of environments and migration mechanisms available to tumor cells.

2.1. Topographic Cues Potentiate Rapid Tumor Cell Migration

Through Tunnel-Like Spaces

An important migration modality in vivo is migration through tunnel-like spaces. Such spaces occur in the interstitial spaces of the tumor stroma (Alexander et al., 2008; Gritsenko et al., 2012; Weigelin et al., 2012), circulatory or lymph vessels (Alexander et
al., 2013), or vasculature of target organs (Alexander et al., 2013; Naumov et al., 1999). Tumor cells predominantly invade along extracellular matrix (ECM) or muscle fibers, or along or within blood or lymphatic vessels (that is, migration occurs both peri-vascularly/lymphatically and intra-vascularly/lymphatically) (Alexander et al., 2013; Lugassy et al., 2007; Lugassy et al., 2014). A growing body of intravital microscopy evidence demonstrates that these tracks are not created solely by tumor-cell mediated matrix remodeling but are found in healthy tissue as well (Weigelin et al., 2012; Wolf et al., 2009). In fact, the ECM encompasses a wide range of protein-free pores ranging from <1-20 µm in diameter (~10-1000 µm² pore area), as well as structures that are not well recapitulated by predominantly homogeneous in vitro gels (Wolf et al., 2009). For example, the human dermis consists of a heterogeneous mixture of 20-50 µm diameter collagen fibers, often with some alignment, and pore sizes of 2-10 µm in diameter (Wolf et al., 2009).

In the context of cancer cell invasion, migration tracks bordered by thick, aligned collagen bundles create “paths of least resistance” for cell migration away from a primary tumor (Alexander et al., 2008; Provenzano et al., 2006). Such migration paths are clinically important. Cell invasion from primary breast tumors is associated with bundled collagen fibers aligned radially to the tumor interface (Provenzano et al., 2006), and such radial alignment is predictive of poor survival independently of other prognostic factors (Conklin et al., 2011). Perivascular spaces and white matter tracks in the brain offer “highways” for glioma cell migration (Cuddapah et al., 2014), and melanoma cells that have extravasated in the brain use blood vessels as guidance structures for continued migration and proliferation (Kienast et al., 2010). In orthotopic MTLn3 breast cancer
tumors in mice, cells adjacent to blood vessels are more migratory than cells elsewhere in the tumor and align with the vessel over time (Kedrin et al., 2008). Interestingly, MTLn3 cells associated with a high occurrence of lung metastases preferentially migrate along collagen fibers in the primary tumor (Sahai et al., 2005). Migration along collagen fibers has also been observed in PyMT-derived primary mammary tumors in mice (Wang et al., 2007) and in xenograft models of primary cancers (TN1 line) (Sharma et al., 2012). Guidance structures are further involved in metastatic colonization. Following escape from a primary tumor, adhesion or occlusion of cells in capillaries leads to tumor cell elongation and migration at potential metastatic sites, including in complex environments such as vessel bifurcations (Yamauchi et al., 2008; Yamauchi et al., 2005; Alexander et al., 2013; Tsuji et al., 2006). Mast cells, macrophages, and cancer-associated fibroblasts in the tumor microenvironment further remodel the extracellular matrix and provide both proteinases and collagen crosslinking to create pro-migratory niches, and they may also be involved in invasion to secondary tissues (Hanahan et al., 2012; Goetz et al., 2011).

Importantly, migration tracks are, in most environments, of the same caliper before and after tumor cell migration, indicating non-destructive migration (Weigelin et al., 2012). This offers an alternative to the notion that cancer cells migrate destructively, by squeezing through and degrading matrices in their invasion path. Instead, it has been demonstrated that preexisting longitudinal channels are physiologically relevant migration environments. These observations could help explain the poor performance of matrix metalloproteinase (MMP) inhibitors in vivo (Coussens et al., 2002), as tumor cells can utilize preexisting migration spaces instead of generating tracks de novo.
Chapter 3

BIOENGINEERING METHODS FOR STUDYING CONFINED MIGRATION

While *in vivo* studies have been important for elucidating the importance of confinement in tumor cell migration and metastasis, the complexity and cost of these models make *in vivo* mechanistic studies difficult. Instead, engineering approaches enable modeling of confined spaces for high-throughput experiments in well-defined microenvironments. To decouple individual factors important for cell migration (e.g., cross-sectional area available for migration, substrate stiffness, ligand density, presence of external gradients, etc.), these studies often involve sophisticated microfabrication and micropatterning techniques. Microfabrication methods allow construction of microenvironments with widths of approximately 10% to >200% of the cell diameter (Balzer et al., 2012; Irimia et al., 2007; Irimia et al., 2009; Kraning-Rush et al., 2013; Mak et al., 2011; Mak et al., 2013; Pathak et al., 2013; Prentice-Mott et al., 2013; Raman et al., 2013; Rolli et al., 2010; Stroka et al., 2014b; Tong et al., 2012; Hung et al., 2013), with independent control of substrate stiffness (Kraning-Rush et al., 2013; Pathak et al., 2012; Ilina et al., 2011), ligand localization (DeForest et al., 2011), and electrical (Huang et al., 2013) and chemical (Irimia et al., 2007; Tong et al., 2012) gradients, or control of cell height, which can be set to an accuracy of ~100 nm (Le Berre et al., 2014). In contrast, pre-conditioning three-dimensional (3D) gels using tumor cells
or fibroblasts with active MMPs creates similar 3D tracks but loses fine control of substrate topography (Fisher et al., 2009; Gaggioli et al., 2007). Methods and devices to create well-defined microenvironments for the study of cell migration are summarized below.

3.1. Fabrication of Microfluidic Devices for Confined Migration Studies

Micropatterned devices capable of presenting confining geometries that mimic the tunnel-like spaces seen in the body are important tools to study cells migrating in confined spaces analogous to those found in vivo. These devices consist of well-defined, pre-formed migration tracks and are most often formed using photolithography. In this process, micron-scale features are patterned on a silicon wafer or glass slide to create a “master” that is used as a replica mold. The features are formed by selective polymerization or degradation of a photoresist upon exposure to UV light. The pattern of light exposure is controlled by a photolithography mask, which contains clear and opaque regions to allow and block the passage of light, respectively. Non-polymerized photoresist is then washed off to expose the desired feature pattern. In most cases, an additional polymer (e.g., polydimethylsiloxane (PDMS) (Tong et al., 2012; Rolli et al., 2010; Breckenridge et al., 2010; Irimia, 2014; Vargas et al., 2014), collagen (Kraning-Rush et al., 2013), polyacrylamide (Pathak et al., 2012; Pathak et al., 2013)) is poured over the microfluidic master and polymerized to form a final device by micromolding. Cells are introduced to such a device for confined migration experiments. This process is illustrated schematically in Figure 3-1.
Figure 3-1. Overview of a typical microfabrication process. A typical microfabrication process involves (A) photolithography to fabricate a master or mold, (B) micromolding to generate a microfluidic device, and (C) cell seeding and imaging to study the effect of the microenvironment on cell behavior. During master fabrication (panel A), a collimated light source (indicated by blue arrows) passes through a photomask containing opaque and transparent regions to cause selective crosslinking of a photoresist (gold layer) that has been spin-coated on a silicon wafer (grey disk). Developing or washing away the non-polymerized photoresist results in a pattern on the wafer. Patterned features have lateral designs defined by the transparent regions of the photomask and heights defined by the thickness of the photoresist layer. A number of polymers can be used to make molds from the master (Panel B). Release of a cured polymer from the mold results in patterned device. Oftentimes, the device is bonded to a glass slide (Panel C), and cells are introduced. For time lapse assays, the final device is imaged on a microscope.
Microfabrication allows a wide diversity of channel sizes and designs to be produced. Typically, microchannels are straight, shaft-like spaces that range from 3-15 µm in height and 3-50 µm in width, although more complex geometries are possible (Prentice-Mott et al., 2013; Scherber et al., 2012; Mak et al., 2014; Mak et al., 2011; Mak et al., 2013; Chen et al., 2015; Heuze et al., 2011). Importantly, these microchannel devices are uniquely able to monitor the real-time migration of cells in controlled 3D environments in the absence of shear stress (Irimia et al., 2007), with the ability to physically confine cells, impose various gradients and pharmacological agents, and control substrate stiffness.

While microfabrication and subsequent micromolding is the most common method used to engineer microenvironments for confined cell migration studies, other techniques have been explored. For example, photocrossliking (Klozin et al., 2010; DeForest et al., 2011) and photodegradation (Kirschner et al., 2013; Ilina et al., 2011) can be used to precisely pattern ligands or create void spaces in 3D gels. Another form of confinement is imposed when microfabricated stamps are used to ink thin stripes or patterns of a polymer on a 2D surface. Cells are confined by adhesions to these stripes and undergo “1D” migration (Doyle et al., 2009; Petrie et al., 2009b; Sharma et al., 2012).

### 3.2. PDMS Microfluidic Microchannel Devices

The majority of microfabricated devices to study cell migration in 3D confinement are composed of polydimethylsiloxane (PDMS), an elastomer that is optically transparent and through which oxygen can diffuse (Sackmann et al., 2014; Berthier et al., 2012; Duffy et al., 1998). PDMS devices for confined migration studies
are typically produced by micromolding PDMS from a patterned silicon wafer (Heuze et al., 2011). In these devices, microchannels arrayed adjacent to cell seeding areas allow for direct, real-time imaging of cell migration under confining conditions and in the absence of shear stress (Irimia et al., 2007). Channels can be coated with a variety of extracellular matrix proteins (Irimia et al., 2009; Tong et al., 2012) and are designed to explore both basic mechanisms of migration in confinement and cell response to external gradients.

3.2.1. Straight PDMS microchannels. PDMS microchannels fabricated to study cell migration during physical confinement are typically straight, shaft-like spaces ranging in cross-sectional area from ~20 µm² to greater than 1000 µm². Early studies of microchannel migration examined the effects of varying channel cross-sectional area on cell migration speed. Irimia and colleagues studied the spontaneous migration of tumor cells through 600 µm-long PDMS microchannels of varying dimensions (3 or 12 µm in height; 6, 10, 12, 15, 18, 25, 30, 50, 75, or 100 µm in width) (Irimia et al., 2009). The migration speed of MDA-MB-231 cells in 3 µm-tall channels peaked at 50 µm/h in 25 µm-wide channels and showed an overall biphasic response to cross-sectional area, while speed was not a function of channel width in the taller microchannels (Irimia et al., 2009). In a similar study, Panc-1 cells plated in the presence of serum entered 150 µm-long fibronectin-coated PDMS channels that were 11 µm tall and 3, 7, or 15 µm wide (Rolli et al., 2010). The Panc-1 cells used in the study were not able to enter 3 µm-wide channels, and only 7% of Panc-1 cells could penetrate 7 µm-wide channels (Rolli et al., 2010). Cell speed was more than doubled in 7 µm-wide channels compared to 2D
surfaces, and cells in microchannels moved in a sliding motion, with small (8%) deviations in cell length (Rolli et al., 2010). It is currently unknown whether cell migration speed is strictly a function the cross-sectional area of the microchannel or if, for example, cell speed is different in 3 µm-wide by 10 µm-tall vs. 6 µm-wide by 5 µm-tall vs. 10 µm-wide vs. 3 µm-tall microchannels.

3.2.2. **Chemical gradients.** The lack of convective flow through high hydraulic resistance microchannels enables formation of chemical gradients between source and sink channels oriented perpendicularly to the microchannels (Irimia et al., 2007). Therefore, these assays are readily adaptable for chemotaxis studies. The stability of the gradient can be improved by incorporating a stream contact zone prior to the microchannels to balance pressure differences between the source and sink channels (Irimia et al., 2007). This design ensures that no pressure difference is present between the microchannel inlet and outlet that would drive convective flow through the microchannels (Irimia et al., 2007). The presence of a gradient establishes a preferential direction for cell transmigration through microchannels (Breckenridge et al., 2010). For example, Tong and colleagues described a microfluidic device through which cells migrate up an FBS chemotactic stimulus through 10 µm-tall channels of width 50, 20, 10, 6, or 3 µm (Tong et al., 2012). HOS cell migration speed was dependent on microchannel width, with speed tending to increase as channel width increased and more cells entering channels as the steepness of the gradient increased (Tong et al., 2012). The device also showed differential migration of metastatic and tumorigenic cells, with MDA-MB-231 cells more likely to enter and exit microchannels than tumorigenic but non-metastatic
MCF-7 cells (Tong et al., 2012); this suggests a possible use of such devices to screen for metastatic cells. Finally, the effect of ECM coating on cell migration was assayed, with migration fastest in collagen I-coated microchannels and slowest in microchannels coated with hyaluronic acid (Tong et al., 2012). A similar device has since been used to study the interplay of protrusions and contractility in confined migration (Hung et al., 2013), demonstrate a migration mode in which microtubules instead of F-actin can drive leading edge protrusion (Balzer et al., 2012), and show the contribution of water flux and cell volume regulation in confined cell migration (Stroka et al., 2014b). Assays reliant on confined chemotactic migration are further operable in the presence of various pharmacological agents, allowing for screening of drug candidates. A device recently constructed by Zhang and colleagues contained 3120 microchambers, each incorporating 10 µm-wide by 10 µm-tall by 400 µm-long microchannels, that allowed for simultaneous screening of 9 pharmacological inhibitors (with multiple technical replicates and seeding densities for each compound) during migration up an FBS gradient (Zhang et al., 2014). This screen revealed differential migration response of SUM-159/Taxol cells to different inhibitors (Zhang et al., 2014).

3.2.3. Voltage gradients. In addition to chemical gradients, cells respond to electrical fields. Huang and Searson tested the effects of combining confinement (20 µm-wide, 80 µm-tall PDMS channels coated with fibronectin) and an electric field applied between two Ag/AgCl electrodes on the motility of NIH 3T3 cells (Huang et al., 2013). While NIH 3T3 fibroblasts aligned perpendicularly to the electric field and migrated toward the cathode when they were unconfined, they aligned and migrated along microchannels
oriented parallel to the field in confinement, suggesting that topographical cues provide a stronger alignment cue than electric fields (Huang et al., 2013). When no electric field was present, cell migration in the microchannels was significantly faster than that on 2D surfaces (Huang et al., 2013). In the presence of a 2.2 V/cm electric field, cells migrated along the channels and toward the cathode, with a persistence significantly greater than when in the absence of a voltage gradient (Huang et al., 2013).

3.2.4. Physical gradients and cell decision making. Modification of microchannel devices to incorporate more complex channel geometries allows for testing of cell response to anisotropic physical gradients or cues. Cells migrating in PDMS microchannels generally continue on their path when encountering an obstacle, although the geometry of the microenvironment and the cell type influences the likelihood of repolarization. MDA-MB-231 breast tumor cells migrating in 15 µm wide, 10 µm tall microchannels that constrict to 3.3 µm in width go through a delay period prior to moving fully through the pores; the delay period increases as the length of the 3.3 µm-wide pore increases (Mak et al., 2013). A percentage of cells encountering such a constriction repolarize (between 14-88% of cells repolarizing, depending upon the cell type and geometry of the constriction) (Mak et al., 2011). When the decision region is made more complex by incorporation of a 90° split to 10 or 3.3 µm-wide, 10 µm-tall channels, over 90% of the cells choose the wider channel, a trend that holds when actomyosin contractility is inhibited by application of blebbistatin (Mak et al., 2014). If the 3.3 µm-wide channel is placed in a path collinear to the original channel, the percentage of cells choosing the wider path drops to 68% and, interestingly, to only 33%
when cells are treated with blebbistatin, suggesting a role of actomyosin contractility in environmental sensing (Mak et al., 2014). Cells migrating through narrow microchannel “mazes” may locally consume growth factors to create gradients that assist in finding the shortest migration path (Scherber et al., 2012), but this strategy may not hold at bifurcations that are asymmetric or larger than the cell body. In some cases, neutrophils migrating through bifurcating microchannels push water to sense the hydraulic resistance of each channel encountered and move into channel with the lowest hydraulic resistance, in the presence or absence of a gradient (Prentice-Mott et al., 2013). If a chemical gradient is present, neutrophil choice to migrate into the shorter channel may also be due to the presence of a steeper gradient in the shorter channel (Ambravaneswaran et al., 2010). Whether such a mechanosensitive strategy dependent on hydraulic resistance can be adopted by tumor cells, which migrate more slowly and rely on the transit of fluid through the cell body for confined migration (Stroka et al., 2014b), is currently unknown.

3.3. Grooved Substrates

Micromachined and microimprinted substrates contain nano- to micron-scale topographies on otherwise planar substrates, typically in the form of parallel grooves. The width, depth, and spacing of these grooves determines whether cells migrate between grooves (Gallego-Perez et al., 2012), analogous to along one side wall of a very tall microchannel, or span grooves to integrate sub-cellular topographic cues (Teixeira et al., 2003; Hamilton et al., 2010; Kim et al., 2009). Cells plated on grooved substrates typically align and migrate along the long axis of the grooves. For example, periodontal ligament fibroblasts align and migrate along 100 nm-tall nanogrooves spaced 500 nm apart, with vinculin-containing adhesions aligning along the grooves 24 h after cell
seeding (Hamilton et al., 2010). Similarly, human gingival fibroblasts plated onto titanium substrates with 3 μm-deep, 15 μm-wide grooves spaced 15 μm apart align actin filaments, microtubules, and focal adhesions along the grooves (Oakley et al., 1993). When the groove spacing is less than the width of the cell, groove depth appears to play a larger role than the distance between grooves. Alignment of corneal epithelial cells over grooves spaced 400-2000 nm apart is significantly greater when the grooves were 600 nm- vs. 150 nm-deep (Teixeira et al., 2003). Confluent monolayers of both epithelial and fibroblast cells also respond to substrate topography, aligning and migrating along 1 μm-wide, 150 nm-deep PDMS grooves spaced 1 μm apart with higher persistence than on flat surfaces (Londono et al., 2014). Interestingly, the topographical cues from the patterned region of the substrate are propagated onto a neighboring flat region of the device to cause oriented migration over a distance of about 9 cell widths, and these propagation distances are not dependent on cell adhesions or actomyosin contractility (Londono et al., 2014). Instead, propagation distances for aligned migration decreased when cell density is decreased, indicating a mechanism reliant on mechanical exclusion (Londono et al., 2014).

### 3.4. Microprinted Lines and Islands

Microcontact printing and its variants are used to laterally constrain cells. By controlling available spreading area, microcontact printing can be used to impose different shapes on either stationary (Thery et al., 2009) or migrating (Maiuri et al., 2012) cells. Microcontact printing is traditionally performed by “inking” an elastomeric stamp with a protein and transferring that protein to a substrate (Alom Ruiz et al., 2007). Non-
stamped regions are then back-filled with a polymer that is non-adhesive to cells (Tan et al., 2004). Recently, other methods have been pioneered. “Stamp-off” approaches allow printing of adjacent, multiple-component patterns on the microscale (Desai et al., 2014). Optical ablation of a non-adhesive polymer followed by back-filling with a cell-adhesive protein allows feature sizes down to ~1 micron in width to be fabricated (Doyle et al., 2009). Selective cross-linking of polymers through photolithography masks also can be used to generate islands of different stiffness or composition on a hydrogel background (Wong et al., 2014b).

3.4.1. Microprinted 1D lines to study cell migration. Lateral confinement to constrain cell migration along a single axis is a useful tool for studying persistent cell migration, the effects of confinement on cell morphology, and intracellular signaling. The resulting migration has been termed “one-dimensional” (1D) migration, as cells are constrained to move back and forth in one direction when the width of the line is less than the width of the cell body. Doyle and Yamada studied cell migration on 1D printed fibronectin lines ranging in concentration from 0.5-1000 µg/ml (Doyle et al., 2009). Cells constrained to linear migration paths moved with significantly greater speed than cells in 2D, and speed was independent of ligand density (Doyle et al., 2009). Uniaxial migration was lost if ECM line width exceeded 5 microns, and migration speed decreased (Doyle et al., 2009). Microcontact printing techniques have also been used to show Golgi and MTOC localization to the rear of the cell during persistent migration of epithelial cells, with repositioning to the new trailing edge upon cell direction change (Pouthas et al., 2008), and that MTLn3E tumor cell migration speed increases on 1D lines when cultured with
bone marrow-derived macrophages (Sharma et al., 2012). *In vivo*, rapid and directional tumor cell migration often occurs upon coupling of tumor cells and macrophages (Sharma et al., 2012). 1D migration assays have recently been used to demonstrate an exponential coupling between cell speed and persistence, where persistence time increased as the actin retrograde flow rate increased (Maiuri et al., 2015). Microcontact printing migration assays can be further integrated with traction force microscopy to probe cell force exertion during lateral confinement (Chang et al., 2013). For example, NIH 3T3 fibroblasts exert lower traction stresses on 10 µm-wide printed lines than on 50 µm-wide printed lines (Chang et al., 2013). It should be noted that this type of migration relies on cell adhesion to the substrate.

### 3.4.2. Microcontact printed islands.

The effects of confined geometry on cell polarization have been extensively studied through the use of microcontact printed “islands” on which cells adhere. Théry and colleagues used microcontact printing to show that cell polarity could be established in different directions in cells with the same overall shape but distinct underlying patterns of adhesion (Thery et al., 2006). Centrosome position in human retinal pigment epithelial cells was maintained at the cell centroid, regardless of the pattern of underlying adhesions, while the nucleus tended to locate toward non-adhesive edges (Thery et al., 2006). When 3T3 fibroblasts were confined and forced to assume elongated morphologies via microcontact printing, cell migration direction upon release occurred along the long axis of the cell (Chen et al., 2013a). Changes in cell spreading area driven by microcontact printed islands can also direct mesenchymal stem cell differentiation (McBeath et al., 2004). This method can be
extended for printing of patterns on polyacrylamide (PA) gels (Rape et al., 2011) or micropost arrays (Tee et al., 2011) to study the effects of confinement on traction force exertion.

3.4.3. **Micropatterned stiffness islands.** Microprinting and micropatterning can be further extended to define rigid islands (consisting of a crosslinked photoresist) in various configurations on a non-adhesive polyacrylamide hydrogel of a given stiffness (Hoffecker et al., 2011; Wong et al., 2014b). NIH 3T3 fibroblasts bridge gaps between adhesive regions on stiff (13 kPa) but not soft (0.8 kPa) gels, with adhesions maturing when the micropatterned island was not deflected toward the spreading cell (Wong et al., 2014b). Confinement itself decreased focal adhesion area (Wong et al., 2014b). Cell spreading across rigid substrates was dependent on Rac1 and Arp 2/3, and probing rate was decreased upon inhibition of Cdc42 or formin homology 2 domain (Wong et al., 2014b). Myosin II-mediated contractility was involved in both rigidity sensing (by pulling on nascent adhesions to test the stiffness of the underlying substrate) and protrusion retraction (Wong et al., 2014b). Indeed, when myosin II was inhibited by blebbistatin treatment, cells were not sensitive to rigidity and spread to occupy islands on soft substrates (Wong et al., 2014b).

3.5. **Vertical Confinement**

Vertical confinement assays made possible by microfabrication techniques enable vertical cell confinement to heights of as little as 3 µm, with an accuracy of 100 nm over large (cm²) areas (Le Berre et al., 2014). In this technique, a pillared PDMS array is lowered over a bed of cells, and cells dispersed between the pillars are vertically confined
to a height defined by the height of the pillars (Le Berre et al., 2014). This is a very high-throughput technique if true 3D confinement is deemed unnecessary and vertical or “sandwich” confinement is sufficient, and it is readily adapted to multiwall plates (Le Berre et al., 2014). Application of this technique has demonstrated how confinement can lead to rupture of the nuclear lamina and modulate gene expression pathways (Le Berre et al., 2012), and how mesenchymal cells can spontaneously switch to a rapid amoeboid migration mode when vertically confined (Liu et al., 2015).

3.6. Patterned Gels

Recent work has extended confinement assays to orthogonally probe the effects of substrate stiffness and microchannel size. These studies are important compliments to 3D gel migration studies because they assay migration through defined microchannels, some of which are non-degradable. This migration regime may be especially important to understand physiological migration through microchannel-like spaces. Both optical and micromolding techniques make fabrication of such structures possible.

3.6.1. Variable pore sizes via controlled polymerization. Randomly dispersed 3D pores can be generated in homogeneous collagen gels by controlling polymerization conditions or using collagen stock with varying telopeptide content. For example, polymerizing rat tail collagen (1.7 mg/ml) at 9°C generates heterogeneous pore sizes (with a median pore cross section of 30 µm²), while polymerization at 37°C results in matrices with a median pore size of only 5 µm² (Wolf et al., 2013). Use of bovine dermal collagen with reduced telopeptide content results in ~5-fold larger pore sizes and reduced matrix elastic modulus at the same overall collagen concentration (Wolf et al., 2013). These techniques
generate large pores that cells can navigate independently of MMP activity (Wolf et al., 2013; Haeger et al., 2014), but they are still often smaller than pores found in collagen matrices in vivo (Wolf et al., 2009). Cell-derived matrices (CDMs) also have local orientation of fibers left by the cells depositing the matrix (Hakkinen et al., 2011), but the spatial organization of the fibers is not controlled. However, these techniques sacrifice fine control of track length and width (particularly in pore size distribution), and other alternatives have been used that provide this control.

3.6.2. Photopatterned gels. Photopatterning relies on light to change or remove defined regions of a hydrogel. Controlling the delivery of light to 2D regions using photolithography masks or 3D regions using focused laser light allows exquisite spatial and temporal control of light-based reactions (DeForest et al., 2011; DeForest et al., 2012). Light-based reactions enable patterning of defined peptides (e.g., for cell adhesion) in 3D, with ~1 um resolution in the x-y plane and ~3-5 um resolution in the z plane (DeForest et al., 2011). For example, ECM proteins and growth factors can be patterned within a 3D matrix to direct cell migration without affecting local topography (Mosiewicz et al., 2013).

Ligand patterning reactions can be coupled with photodegradation to create void spaces in 3D or degrade portions of the surface of a gel (Kloxin et al., 2010). Two-photon laser microsurgery has been used to ablate regions within a collagen gel, leading to 3D tracks within the matrix that are bordered by degradable collagen (Ilina et al., 2011). Such a scheme was used to study the invasion of multicellular spheroids of mouse mammary tumor (MMT) cells embedded within dense fibrillar collagen (Ilina et al.,
Cells were able to invade tracks with cross-sectional areas of 9, 25, 100, 400, and 900 µm$^2$ in the absence of matrix metalloproteinase activity and enlarged narrow tracks over time by an outward pushing mechanism (Ilina et al., 2011). Engineered hydrogels that degrade upon exposure to light offer an alternative to photoablation to define void spaces within a 3D matrix. Degradation of a hydrogel from the top surface has been used to create grooves in an otherwise flat hydrogel substrate (~1-5 µm depth, 5 µm width, spaced 5 µm apart) (Kirschner et al., 2013). Photodegradation has also been used to pattern cell culture wells in hydrogels to study the differentiation of mouse alveolar type II cells, with additional rectangular channels formed after several days of cell growth via two-photon photopatterning to make connections between cell clusters in real time (Kloxin et al., 2012). Orthogonal techniques for controlling ligand concentration and microtrack geometry have, for example, been used to study 3T3 fibroblast migration through microchannels created around a fibrin clot; when presented with a choice between channels functionalized with RGD peptide and those without, cells only migrated into channels with the peptide (DeForest et al., 2011). Such approaches can also be used to temporally and spatially release cells or groups of cells from certain regions of a 3D gel for sub-population sampling (DeForest et al., 2011).

3.6.3. Micromolded collagen and polyacrylamide hydrogels. Instead of using PDMS to mold from a patterned master, other polymers can be used. This allows orthogonal control of substrate stiffness and microchannel geometry (Pathak et al., 2012) and can be performed with either degradable (Kraning-Rush et al., 2013; Carey et al., 2015) or non-degradable (Pathak et al., 2012) polymers. Microtracks (10 µm wide, 20 µm tall, and 300
µm long) formed in collagen polymerized against a PDMS mold can be fabricated in 1.5, 2, 3, and 5 mg/ml collagen type I (Kraning-Rush et al., 2013). The presence of microtracks increases the migration speed of MCF10A and MDA-MB-231 cells compared to unpatterned 3 mg/ml collagen gels (Kraning-Rush et al., 2013). Interestingly, migration speed is not a function of collagen concentration in these tracks (Carey et al., 2015). Polyacrylamide troughs that confine cells on three sides can also be molded from microfabricated masters. The migration speeds of U373-MG human glioma cells through microchannels of with 10, 20, or 40 µm and stiffness ranging from 0.4 to 120 kPa is biphasic in wide (20-40 µm) channels but increases monotonically with stiffness in 10 µm-wide troughs (Pathak et al., 2012).

3.7. Conclusion

Advances in chemistry and microfabrication techniques have enabled the creation of many tools to study cell behavior in confinement. The migration mechanisms that use of these tools have elucidated are summarized in the next chapter.
Chapter 4

MECHANISMS AND COMPENSATION STRATEGIES FOR CONFINED MIGRATION

Bioengineering tools have been used extensively to explore how confinement and mechanical forces influence cell topology, signaling, and migration mechanisms. Importantly, the wide variety of migration mechanisms available to tumor cells may impact the ability of targeted treatments to inhibit tumor cell migration. Here, we summarize the migration mechanisms that in vitro models of tunnel-like migration spaces found in vivo have revealed. These mechanisms offer exciting potential targets for the abrogation of tumor cell migration.

4.1. Topographical Features Elicit Spontaneous Cytoskeletal Alignment and Persistent Cell Migration

Cells encountering topographical features spontaneously align along the features in a process called contact guidance. During cell spreading along grooved substrates, microtubule alignment typically precedes alignment of the actin cytoskeleton, though cells can compensate to align to topographic features in the absence of either microtubules or actin filaments (Oakley et al., 1993; Oakley et al., 1995; Oakley et al., 1997). In microchannels wider than the cell body, cells elongate along one channel side.
In narrower microchannels, tumor cells are in contact with all four channel walls and generally take on a pill-like morphology, with nuclear elongation, cortical actin alignment along the long axis of the microchannel, suppression of stress fibers, and increasingly diffuse focal adhesion protein staining as the width of the channel decreases (Balzer et al., 2012; Pathak et al., 2012). In very narrow channels, F-actin is often enriched at the cell poles (Tong et al., 2012; Balzer et al., 2012). A similar reduction in focal adhesion size and homogenous distribution of adhesion proteins occurs on narrow microcontact printed lines, and actin and microtubules orient parallel to the printed lines, with stabilized microtubules accumulating in an anterior bundle (Doyle et al., 2009). Loss of stress fibers and homogeneous vinculin distribution are also seen in cells migrating in vertically confining “sandwich” assays, with patches of myosin II accumulating at the cell rear; these cells often migrate with a morphology similar to that seen in migrating neutrophils (Liu et al., 2015). Morphological characteristics of confined cells are summarized in Table 4-1.

### Table 4-1. Morphological characteristics of confined cells

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<th>Morphological characteristics of confined cells</th>
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<tr>
<td>Suppression of stress fibers and actin distribution around cell periphery</td>
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<td>Alignment of cytoskeletal features (actin, microtubules)</td>
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<td>Homogeneous (as opposed to focal) expression of adhesion proteins</td>
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<td>De-coupling of migration speed and ligand density</td>
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<td>Nuclear deformation</td>
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Cell polarization upon confinement leads to spontaneous and persistent migration of numerous tumor cell lines through microchannels with cross-sectional areas ranging from 18-1200 µm² (Irimia et al., 2009; Rolli et al., 2010), with displacements greater than those predicted by 2D random walks (Mak et al., 2013). Persistence generally increases as microchannel cross-sectional area decreases (Desai et al., 2013). Modifying microchannels to incorporate turns and variations in width can elicit even more persistent migration, even in cells overexpressing Rac1 that change direction often in 2D environments (Ko et al., 2013). Vertical confinement also induces rapid and persistent migration of tumor, epithelial, and immune cells (Liu et al., 2015). Increases in persistence in contact-guided cells are due, at least in part, to a decrease in the occurrence of nascent protrusions away from the cell poles (Riching et al., 2014), possibly due to the action of myosin II-mediated contractility to minimize cell-surface curvature (Elliott et al., 2015).

Confinement further influences cell polarization by inducing persistent localization of organelles. In 36 µm² microchannels, MDA-MB-231, PC3, PC3M, and MDA-MB-435 cells move fastest and most persistently upon anterior localization of the mitochondria with respect to the nucleus (Desai et al., 2013). Generation of large mitochondria following genetic intervention with mitochondrial fission and fusion proteins causes cells to move more slowly and less persistently (Desai et al., 2013). Epithelial cells confined to 6 µm-wide printed fibronectin lines elongate and migrate with preferential Golgi localization behind the nucleus, with a reduction in posterior localization as line width increases to 30-40 µm (Pouthas et al., 2008). The MTOC is positioned near the Golgi complex on 6 µm-wide lines, and both organelles reposition
upon a directional change (Pouthas et al., 2008). However, when cells are confined and then released, the direction of migration is set by the axis of the cell instead of by the position of the Golgi, so cytoskeletal and membrane organization are likely more important than organelle position in the initiation of polarized migration (Chen et al., 2013a). Overall, organelle positioning likely interacts with other drivers of cellular polarization to cause persistent migration in confinement.

Importantly, contact guidance of cells along physical features also affects immune cell trafficking relevant to cancer immunotherapy. High ECM protein density in tumors drives trafficking circumferentially to tumor cells, and treatment with collagenase significantly increases the number of T cells in the vicinity of tumor cells (Salmon et al., 2012). Similarly, T cell localization to a tumor is a function of collagen density instead of chemokine level in pancreatic cancer, even though T cell chemokines are overexpressed in pancreatic cancer (Hartmann et al., 2014). Indeed, confined migration is important not only in understanding the migration of tumor cells but also in devising strategies for tumor immunotherapy. It is worth noting that microchannel models can provide important platforms for studying the characteristics and mechanisms of the immune response (Chabaud et al., 2015; Prentice-Mott et al., 2013; Hamza et al., 2015; Hamza et al., 2014; Boneschansker et al., 2014; Jones et al., 2014).

4.2. Nuclear Confinement Modulates Tumor Cell Migration and Division

Numerous reports suggest that the nucleus plays a rate-limiting role in confined migration, particularly in highly confining microenvironments. This is likely clinically
important, as lamins (the primary components of the nuclear lamina and prime
determinants of nuclear mechanics) are aberrantly expressed in a number of cancers (Ho
et al., 2012). In collagen gels, limits on nuclear deformation prevent tumor cell migration
through pore sizes smaller than ∼7 µm², an area approximately 10% of the nuclear cross-
sectional area (Wolf et al., 2013). That said, there is likely an interplay between nuclear
stiffness and pore size in determining the limits of confined migration. Cells depleted of
lamin A/C more quickly pass through 10 µm² constriction points than cells with wild-
type lamin expression (Davidson et al., 2014), and knockdown of the nuclear envelope
protein lamin-A decreases nuclear stiffness and increases transmigration of A549 lung
carcinoma cells through 3 µm-wide (3% of projected nuclear area) but not 8 µm-wide
(23% of projected nuclear area) pores in a Transwell assay (Harada et al., 2014). Further,
in xenograft studies using A549 cells, cells at the periphery of the tumor show lower
lamin-A:B ratios and greater nuclear elongation, suggesting that nuclear flexibility
promotes confined migration in vivo (Harada et al., 2014). Indeed, transmigration of
MDA-MB-231 cells through microfabricated pores 25 µm in length and 50 µm in height
is dependent on contractility-driven nuclear deformation below a characteristic cross-
sectional pore area, with transmigration slowed in 6 µm-wide pores upon blebbistatin
treatment but unchanged in 8 and 12 µm-wide pores (Breckenridge et al., 2010). MDA-
MB-231 cells show a similar reduction in transmigration through 5 µm tall
microchannels as channel width decreases from 12 µm to 4 µm (Fu et al., 2012).
Inhibition of tight chromatin packing to heterochromatin via MTA treatment further
reduces transmigration, presumably due to defects in nuclear reshaping required to
squeeze the nucleus through a pore (Fu et al., 2012).
In addition to the fundamental role of the nucleus in confined migration, confinement can itself drive cell division defects and changes in gene expression, which could accelerate tumorigenesis in the stiffer, compressive environment of a tumor (Potapova et al., 2013; Padera et al., 2004). Division of taxol-resistant MDA-MB-231 cells confined at an interface between 15 and 3 μm-wide channels results in asymmetric daughter cell sizes (Mak et al., 2013). Vertical confinement induced by a short PDMS roof (3-7 μm in height) also increases the number of aberrant divisions (i.e., divisions resulting in three or more daughter cells), increases differences in volume between daughter cells and time required for division, and increases the rate of cell death (Tse et al., 2012; Kittur et al., 2014). Vertical confinement ruptures the nuclear lamina and induces differential expression of genes involved in inflammation, stress response, and membrane synthesis, specifically those involved in DNA damage response and in the NFκB pathway (Le Berre et al., 2012). Similar changes in gene expression have been observed upon confinement within microgrooves (Dalby et al., 2003) and on microcontact printed islands (Thomas et al., 2002). For example, the nucleus elongates and aligns along microgrooves in fibroblasts plated on 12.5 μm-wide, 2 μm-deep grooves etched in quartz slides, and a number of genes are upregulated 24 hours after plating (Dalby et al., 2003). Interestingly, many of these genes were then downregulated compared to flat controls five days after seeding, including genes involved in Rho GTPase signaling and microtubule organization (Dalby et al., 2003). Cells confined to microcontact printed islands designed to cause nuclear elongation synthesize more collagen I and express higher levels of osteocalcin mRNA four days after confinement than cells of similar area but lacking nuclear elongation (Thomas et al., 2002). Even
isolated nuclei can respond to force, not through chomatin or nuclear actin responses but by using a pathway requiring an intact nuclear lamina and the inner nuclear membrane protein emerin (Guilluy et al., 2014). These results suggest that numerous types of confinement are important regulators of cell division as well as migration.

4.3. Tumor Cells Migrate Efficiently Through Microtracks

Independently of MMPs

Mounting evidence suggests that tumor cells can migrate in vivo in the absence of MMPs, which degrade the ECM. For example, A431 and MTLn3E cells move efficiently upon treatment with the broad spectrum MMP inhibitor GM6001 in orthotopic xenograft tumors in mice (Wyckoff et al., 2006). To study the requirements of matrix degradation for tumor cell migration in vitro, photoablation (Ilina et al., 2011) or micromolding (Kraning-Rush et al., 2013) techniques are used to create well-defined tracks in degradable collagen gels. Tracks in 3D matrices can also be created by migrating “leader” cells that express MMPs (Kraning-Rush et al., 2013; Fisher et al., 2009; Gaggioli et al., 2007), but this technique sacrifices precise control of track width, geometry, and ligand coating concentration. MCF10A breast epithelial and MDA-MB-231 breast adenocarcinoma cells are more invasive along tracks patterned in a collagen gel than in unpatterned gels (Kraning-Rush et al., 2013). When MMPs are inhibited using the broad-spectrum MMP inhibitor GM6001, cells are still able to invade into the pre-formed tracks, but collective invasion in non-patterned 3D gels is inhibited (Ilina et al., 2011). Knockdown of MT1-MMP via siRNA significantly decreases MDA-MB-231 speed in 3D collagen matrices but not along microtracks (Kraning-Rush et al., 2013).
Similar results have been seen upon addition of cells and GM6001 to ECM gels previously remodeled by MMP-producing cells (Fisher et al., 2009). Interestingly, migration speeds of cells treated with GM6001 through homogeneous collagen gels are recovered as pore sizes increases (Wolf et al., 2013), and cell invasion from tumor spheroids is not abrogated by GM6001 in collagen gels with 24 µm\(^2\) (but not 8 µm\(^2\)) median pore size (Haeger et al., 2014). Together, these results suggest the existence of robust migration mechanisms in the absence of MMPs, provided that tracks and pore openings of sufficient size are available.

4.4. The Complex Roles of Actomyosin Contractility and Adhesions in Confined Migration

Experiments have suggested various roles for cell-substrate adhesions and actomyosin contractility during confined migration. In environments with pore sizes smaller than the cell nucleus, it is likely that cell contractility and adhesions are required for track formation but dispensible if tracks are already formed. *In vivo*, metastatic MTLn3 cells injected orthotopically into mice deform collagen fibers at the tumor margins, while non-metastatic MTC tumors do not, and ROCK inhibition alters myosin light chain localization and reduces cell motility (Wyckoff et al., 2006). In vivo, A375M2 cells migrate by blebbing when β1 integrin is knocked down (Tozlouoglu et al., 2013). In collagen gels, inhibition of β1 integrin adhesion or ROCK-mediated contractility impairs migration through gels with pore sizes of ~20 µm\(^2\) but not 55 µm\(^2\) (Wolf et al., 2013). Fibroblast track generation in a mixed ECM gel requires integrin α3, integrin α5-Rho-ROCK-MLC-driven matrix remodeling, and MMP activity, but Rho/ROCK contractility
is not required for squamous cell carcinoma cells to follow the fibroblasts into the 10-20 µm-wide tracks created (Gaggioli et al., 2007). MDA-MB-231 cells seeded in collagen gels align collagen fibers in a Rho-dependent manner, but Rho and ROCK are not required for migration if the matrix is already aligned (Provenzano et al., 2008). Similarly, the migration speed of MDA-MB-231 cells migrating through microtracks preformed in collagen gels is not affected by treatment with a function-blocking β1-integrin antibody or the inhibitors Y27632, ML-7, or CT04, and a similar fraction of cells can migrate (compared to control) even after blebbistatin treatment (Carey et al., 2015). In 1.5 mg/ml collagen gels, the fraction of motile cells is drastically decreased when these contractility effectors are inhibited (Carey et al., 2015). β1 integrin is required for HT1080 cells to form tracks in collagen gels (Wolf et al., 2007), and inhibition of ROCK with Y-27632 or ROCK-1/2 knockdown inhibits tunnel formation (Fisher et al., 2009). However, Y27632 does not block motility of HT1080 cellls in collagen gels once tunnels are formed (Fisher et al., 2009). Therefore, it appears that both adhesions and cell contractility are crucial for track creation in matrices with pores smaller than the cell body, but that compensation strategies are available for migration in the absence of adhesions and actomyosin contractility when these tracks are already present.

In microchannel models, confined (30 µm² track cross-sectional area) migration of MDA-MB-231 breast adenocarcinoma cells is not affected by the addition of pharmacological inhibitors of contractility (Balzer et al., 2012). These cells also migrate efficiently in the presence of function-blocking antibodies for β1 integrin (Balzer et al., 2012). Confined migration is accompanied by a reduction in cellular traction forces, which are unchanged upon actomyosin inhibition or activation in 40 µm² but not 200 µm².
microchannels, indicating a reduced role for these forces in confined migration through open channels (Raman et al., 2013). Similarly, migration speed through confining microtracks in collagen gels does not correlate with phosphorylated-myosin light chain levels, though force exertion does, suggesting that force exertion is not directly related to speed during confined migration through tracks (Carey et al., 2015). Furthering the supposition that actomyosin contractility is, at least in some cases, dispensable for confined migration is the observation that migration in confinement can occur even when F-actin polymerization is inhibited via treatment with Latrunculin A (Balzer et al., 2012; Stroka et al., 2014b). To date, the only model capable of explaining this phenomenon is the Osmotic Engine Model, which predicts migration independently of actomyosin contractility and posits that the polarized uptake and expulsion of water at the leading and trailing edges of confined cells causes migration in a jet-like fashion (Stroka et al., 2014b). Indeed, interference with aquaporins or osmotic shock to modulate water uptake inhibits or mediates, respectively, the confined migration of tumor cells (Stroka et al., 2014b). The reduced dependence of cell speed on ligand density during confinement (Doyle et al., 2009; Carey et al., 2015) may be due to the reduced adhesion size observed in confined migration.

Conversely, actomyosin contractility, but not cell-substrate adhesions, is essential in amoeboid migration, where cells produce blebs to drive forward movement. Such migration occurs in tumor cells in vivo, and both a computational model and in vitro experiments suggest that bleb-based migration is most efficient in confinement and in regimes of low adhesiveness (Tozluoglu et al., 2013). Adhesion-independent amoeboid migration in confinement has been observed repeatedly in tumor (Liu et al., 2015) and
immune cells (Liu et al., 2015; Malawista et al., 1997; Lammermann et al., 2008). Polymorphonuclear leukocytes “chimney” between glass coverslips upon treatment with EDTA or functional blocking of integrins (Malawista et al., 1997), and leukocytes can move in three dimensions in the absence of integrins if myosin II contraction is intact (Lammermann et al., 2008). Importantly, confinement allows exertion of force on surrounding substrates in the absence of adhesions and potentiates bleb-based migration of a number of cell types (Liu et al., 2015). Alignment of myosin traction forces in confining polyacrylamide channels leads to increasing migration speed of U373-MG human glioma cells as channel stiffness is increased, but treatment with blebbistatin inhibits traction polarization and leads to biphasic relationships between speed and stiffness in both confined (10 µm-wide) and unconfined (≥20 µm-wide) polyacrylamide troughs (Pathak et al., 2012). Inhibiting contractility also decreases the speed of A375-SM melanoma cells through 6 µm- and 3 µm-wide, 10 µm-tall PDMS microchannels (Hung et al., 2013). Fibroblasts migrating in linearly elastic 3D matrices can also use a pressure-based lobopodial migration mode that is dependent on RhoA-ROCK-myosin II mediated contractility (Petrie et al., 2014). These results suggest that, for some cell types, contractility is needed to align contractile forces and maximize migration speed in confinement (Pathak et al., 2012).

We can speculate that confined cells may migrate using a number of different mechanisms, including a blebbing amoeboidal mode that is highly dependent on cortical contractility and another driven by protrusions and in which the reduction in energy required to push through a stiff actin cortex may overcome any deficiencies caused by a reduced ability of the cell to contract. Indeed, highly confined cells in low-adhesion
environments can migrate rapidly upon Y-27632 treatment in a manner that is dependent on fast actin retrograde flow in a small protrusive region at the cell front, while these cells adopt a blebbing mechanisms dependent on high levels of contractility when the contractile pathway is intact (Liu et al., 2015).

4.5. Cellular Protrusions Optimize Confined Migration

Cellular protrusions work in parallel with contractility to optimize migration through confined spaces. In situations where matrix degradation is coupled with cell migration, actin-based protrusions are particularly important. Mena<sup>INV</sup>, a cytoskeletal regulator protein that leads to enhanced actin polymerization and protrusions, is upregulated in migratory subpopulations isolated from human breast tumors orthotopically grown in mice (Patsialou et al., 2013). Other genes involved in actin-based protrusions, notably Cdc42, are also routinely upregulated in migratory cells isolated from bulk tumors (Patsialou et al., 2013; Wang et al., 2007; Wang et al., 2004). Furthermore, knockdown of Cdc42 or its effector kinases MRCKα and MRCKβ prevents squamous cell carcinoma cells from following fibroblasts into tunnels created in ECM gels (Gaggioli et al., 2007), and knockdown of Cdc42 or the Cdc42 effectors IQGAP-1, MRCKβ, and Pak4 inhibit tunnel formation by HT1080 cells in collagen gels (Fisher et al., 2009). However, the role of Cdc42 in microchannels has not been studied.

In confining microchannels, CHO-α4Y991A cells have higher Rac1 activity and migrate more slowly than CHO cells expressing wild type α4 integrin, but migration speed is recovered upon treatment with the Rac inhibitor NSC23766 (Hung et al., 2013). A375-SM melanoma cells treated with NSC23766 migrate faster in 3 µm-wide by 10 µm-tall microchannels than control cells (Hung et al., 2013). In contrast, Rac inhibition
slows the migration of MCF10A cells through 25 μm-tall polyacrylamide microchannels with widths of 10 μm and 40 μm that range in stiffness from 0.4-120 kPa (Pathak et al., 2013). The role of Rac in confined migration may not be driven directly by effects on cellular protrusions but instead through a crosstalk with the cell contractility pathway, which promotes confined migration when Rac activity is inhibited (Hung et al., 2013). Importantly, actin may be remodeled solely in response to external force, without involving other intracellular signaling pathways. For example, experimental and in silico results suggest that extensional but not compressional force promotes Arp2/3-mediated actin filament branching, further implicating that confinement drives the direction of cell protrusions (Risca et al., 2012). Acellular solutions containing actin filaments are also able to form an actin ring that orients in the plane perpendicular to compressive forces generated by two silicone-coated coverslips spaced approximately 4.5 μm apart (Miyazaki et al., 2015). Inhibition of microtubule polymerization in either narrow microchannels (Balzer et al., 2012) or 1D printed lines (Doyle et al., 2009) significantly reduces migration speed and directionality.

4.6. Confinement Mediates Collective-to-Single Cell and Mesenchymal-to-Amoeboid Transitions

The plasticity of tumor cell migration under confining conditions is perhaps best illustrated by transitions of cell populations between collective and single cell migration, or of single cells between mesenchymal and amoeboid migration modes (Friedl et al., 2011). For example, topographical barriers cause dispersal of cells from a collective sheet (Wong et al., 2014a). MCF-10A breast epithelial cells expressing Snail-6SA scatter
into a population of fast-moving mesenchymal cells with high vimentin expression that migrate individually into an array of 10 µm x 10 µm x 10 µm (height x diameter x spacing) posts, while a collective front with high E-cadherin expression exists in the cell seeding area and initial portion of the micropost array (Wong et al., 2014a). Interestingly, the mesenchymal subpopulation shows less reduction in migration and proliferation upon treatment with Rsk inhibitors BID-1870 and U0126 than the epithelial subpopulation (Wong et al., 2014a). In collagen gels, MV3 melanoma and HT1080 fibrosarcoma cells transition from single-cell to collective migration modes as matrix pore size decreases (Haeger et al., 2014). Collective migration is dependent on MMP function, as track generation is required at high collagen density (Haeger et al., 2014). Interestingly, this switch is not affected by changes in ligand density or matrix stiffness but is instead driven solely to the physical size of the pores in the gel (Haeger et al., 2014). Confinement to microcontact-printed lines of various widths also mediates the mode of collective migration. The collective migration speed of MDCK cells increases upon confinement to 20 µm-wide fibronectin strips, where cells move with push-and-pull force patterns instead of as a single sheet under tension by leader cells at the front (Vedula et al., 2012). This type of migration is mediated by intact cell-to-cell contact and actomyosin contractility (Vedula et al., 2012).

At the single-cell level, imposing confinement can induce a switch from amoeboid to mesenchymal migration across a wide variety of cell types, including cancer cells. A subfraction of cells vertically confined in a sandwich assay, and under conditions of low adhesion, move much more quickly and persistently than cells migrating mesenchymally on 2D surfaces by utilizing a “stable-bleb” migration mechanism (Liu et
al., 2015). This is accompanied by a dense actin cortex at the cell rear (uropod) but not the cell front, patches of myosin II most at the cell rear, and very fast retrograde flow of actin filaments and myosin II in the central part of the cell (Liu et al., 2015). This migration mode also occurs in zebrafish embryos and is dependent on high contractility (Liu et al., 2015; Ruprecht et al., 2015).

4.7. Key Differences in Confined Migration Assays

While the study of cell migration in confinement has pointed to common and important morphological characteristics of confined cells (Table 4-1), it is important to note differences in the migration modes enabled by each type of confinement. First, confinement enables cells to push against their environment and apply forces in the absence of adhesions (Liu et al., 2015). These adhesion-independent forces are physiologically important. Blebbing migration of A375M2 melanoma cells occurs in vivo, even when β1 integrin is knocked down (Tozluoglu et al., 2013), and leukocyte migration speed within lymph nodes is not reduced upon integrin knockdown (Lammermann et al., 2008). While 1D confinement assays recapitulate morphological characteristics of 3D migration (namely reduced adhesion size and suppression of stress fibers) (Doyle et al., 2009), they are dependent on adhesions, as cell sticking to patterned surfaces is required. Cells can deadhere from these lines under conditions of high contractility (Doyle et al., 2009). Therefore, although these assays provide confinement, they thus miss important mechanisms of cell motility.

Furthermore, vertical confinement in sandwich-type assays lacks the full 3D confinement possible in PDMS microchannels or patterned hydrogels. It is currently unknown whether cell migration in microchannels is equivalent to “chimneying” between
parallel planes, or whether cells in microchannels actually exert outward forces on all four walls of a microchannel. Expansion of channel size in patterned hydrogels suggests that force exertion occurs around the entire cell periphery (Ilina et al., 2011). Importantly, whether microchannel and vertical confinement assays induce the same migration mechanisms is currently unknown. It is possible that in very wide or very tall microchannels, only two parallel channel walls are “used” by the cell to move. When confinement increases, cells may adopt alternative migration strategies, such as osmotically driven migration (Stroka et al., 2014b). Similarly, even among microchannel assays, the use of degradable vs. non-degradable polymers to create the microtracks may yield important mechanistic differences. For example, while treatment with blebbistatin does not affect migration speed of tumor cells in non-degradable PDMS (Balzer et al., 2012) or polyacrylamide channels, it decreased migration speed in collagen microtracks (Carey et al., 2015). It will be important to test migration mechanisms under different types of confinement to discover the generality of each of these mechanisms.
Chapter 5

LENS-FREE IMAGING FOR TIME LAPSE CELL MOTILITY ASSAYS

5.1. Introduction

Scientists have imaged living cells since the first observations of motile cells by Leeuwenhoek in the 17th century (Dunn et al., 2004). Time lapse analysis of biological process is essential for understanding dynamic and complex biological processes and has been growing in use and importance (Coutu et al., 2013; Dunn et al., 2004; Frigault et al., 2009; Paddock, 2001; Stephens et al., 2003). Importantly, the confined migration assays discussed above are dependent on time lapse analysis for capturing the point-to-point location and speed of motile cells. However, technical challenges in combining complex imaging systems capable of generating data at the needed temporal and spatial resolution with environments amenable to cell survival limit the use of such assays in some research and most clinical settings.

To address these issues, researchers are developing compact and inexpensive lens-free imaging (LFI) systems based on the principle of digital holography (Isikman et al., 2011). Holography was introduced by Dennis Gabor in 1948 (Gabor, 1948) and resulted in digital holography after direct recording of the Fresnel holograms with charge coupled devices by Schnars and Jüptner in the early 1990s (Schnars et al., 1994). In these systems, the objective is replaced with mathematical calculations, greatly simplifying the optical setup. Despite these advantages, the use of lens-free techniques for imaging
biological processes is in its infancy. Growth of microvasculature networks from endothelial cells cultured in a 3D fibrin matrix has been measured over 24 mm$^2$ with an approximately 1.5 µm spatial resolution, showing the promise of LFI for capturing large areas and depths of focus without mechanical scanning (Weidling et al., 2012). These images were, however, static and captured following microvasculature growth. The low capital costs associated with LFI make it more attractive for high-throughput, time-lapse applications. For example, compact, inexpensive LFI assemblies placed in a cell culture incubator revealed that random motility of NIH 3T3 fibroblasts on polyacrylamide gels of 0.4, 4, or 40 kPa stiffness increased with increasing gel stiffness for ECM coatings of fibronectin and collagen type IV (Pushkarsky et al., 2014). However, this method failed to resolve the morphology of the cells, and results were not compared directly with those captured using established phase contrast methods to ensure that tracks were accurate and not distorted by the in-line holographic imaging method. Additionally, cells were imaged in 2D environments, which do not recapitulate the complex 3D topography of the body as closely as 1D or 3D confined migration assays (Doyle et al., 2009; Stroka et al., 2014a; Stroka et al., 2014c). Extending LFI for use in these assays would greatly improve the translational relevance of results generated with the technique.

Therefore, we worked to benchmark the lens-free imaging technique as a time-lapse live cell imaging tool. We built and validated a lens-free imaging (LFI) platform based on in-line digital holography. The system captures a large field of view in a single image at high resolution, eliminating the need for translating microscope stages. Furthermore, it is housed in a common cell culture incubator, so stage-top incubation setups are not required. Three typical motility assays were imaged side-by-side using
both with LFI and conventional phase contrast microscopy: random motility, confined motility on 1D microprinted lines, and confined motility in 3D microchannels. Comparisons of the results obtained with both imaging techniques were used to benchmark the LFI system. The motility assays were chosen in such a way that they accounted for 1D, 2D, and 3D cell migration, taking into account the importance of microenvironmental topography on cell response (Stroka et al., 2014a). Results were generated for two breast adenocarcinoma cell lines: non-metastatic MCF7 cells and metastatic MDA-MB-231 cells.

5.2. Materials and Methods

5.2.1. Lens free imaging (LFI) setup. The LFI technique is based on the principle of in-line digital holography. A picture of the set-up and schematic representation of the principle can be found in Figure 5-1. In-line holography was chosen for cell migration assays as the imaged objects (cells) are transparent, avoiding the more complex optics needed to maintain a separate reference beam. In order to improve the image quality, multiplexing of holograms was used, i.e., for each image, holograms were recorded at 4 different wavelengths (639, 645, 656, and 664 nm) for iterative phase retrieval. A CMOS chip was used to record the interference patterns instead of a CCD, resulting in faster and less energy-intensive image acquisition. Due to the small size of the set-up, experiments were run inside a common cell culture incubator maintained at 37°C and 5% CO₂ (Fig. 5-2).
Figure 5-1. Lens-free imaging (LFI) principle of operation, setup, and characteristics. (A) Principle of operation of holographic in-line LFI. A collimated light source sent through a pinhole travels a distance $\Delta D$ before encountering a transparent object. The non-interacting reference wavefront and the object wavefront travel a distance $D$ to a sensor (in this case, a CMOS chip), creating an interference pattern that is read by the sensor. The interference pattern is then reconstructed to create an image of the object. (B) Photograph of the LFI platform. (C) Field of

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43
view (6.4 mm x 4.6 mm) obtained with a single LFI image. Scale bar represents 1 mm. (D) Field of view obtained with a single phase contrast microscope equipped with a 10x objective. Scale bar represents 100 µm. Morphology of single cells was clearly visible using both (E) LFI and (F) phase contrast microscopy following digital zoom in the images shown in panels (C,D). Images are shown to the same scale. Scale bar represents 50 µm. In (C-F), MDA-MB-231 cells spread on a collagen type I-coated glass slide are shown.
Figure 5-2. LFI platform setup. The LFI platform consists of components kept outside and inside of a common cell culture incubator. Programmable laser controllers, laser temperature controllers (which set the wavelength of light used), the lasers (housed in a box), and a computer are set up near an incubator. A fiber optic cable and cables controlling the sensor and transmitting the sensor output to a computer are fed inside the incubator. Incident light from the fiber optic cable is transmitted through the sample, which is placed on a platform over a CMOS chip.
5.2.2. Phase contrast microscopy setup. An inverted Nikon Eclipse Ti microscope (Nikon, Tokyo, Japan) with automated controls (NIS-Elements, Nikon) was equipped with a temperature- and CO₂-controlled stage-top live cell incubator (Tokai Hit, Japan) mounted on a software-controlled motorized stage (NIS-Elements, Nikon). Migrating cells were visualized every 10 min with an Andor Clara DR-1261 camera head (Andor Technology, Belfast, United Kingdom) and a 10x/0.45 NA Ph1 objective for the duration of each live cell experiment (8-10 hours). In each experiment, the motorized stage was programmed to capture 35-45 fields of view. The area of each field of view was ~900 µm x 670 µm.

5.2.3. Master fabrication. Microfluidic devices used for microchannel migration assays and as templates for microcontact printing were fabricated using standard photolithography, as described previously (Balzer et al., 2012; Hung et al., 2013; Stroka et al., 2014b; Tong et al., 2012). Briefly, mechanical grade silicon wafers (University Wafer, South Boston, MA, USA) were cleaned with acetone and isopropanol, dehydrated (200°C for 10 min), and spin coated with SU-8 3010 (Microchem, Newton, MA, USA) to achieve a film thickness of 10 µm. The wafer was soft baked on a hot plate (temperature ramped from room temperature to 95°C at 1°C/min and held at 95°C for 5 min) to drive off solvent in the photoresist. The wafer was exposed through a photomask (Photoplot Store, Colorado Springs, CO, USA) defining the microchannels at an energy of 170 mJ/cm² using an EVG620 mask aligner (EVG, Austria). The wafer was post-exposure baked (3 min at 65°C, 5 min at 95°C), and the photoresist was developed using SU-8 developer (Microchem). The developed wafer was rinsed with isopropanol, dried, and
hard baked (10 min at 200°C). The photoresist layer defining the inlet and outlet channels of the device was then deposited by spinning a 50 µm-thick layer of SU-8 3025 (Microchem) over the patterned wafer. This layer was soft baked (temperature ramped from room temperature to 95°C at 1°C/min and held at 95°C for 15 min) prior to exposure at 250 mJ/cm² through an aligned photomask defining the inlet and outlet channels of the device. The wafer was post-exposure baked (3 min at 65°C, 5 min at 95°C), developed, and cleaned with isopropanol. The completed wafer was hard baked (10 min at 200°C) and treated with fluorosilane [(tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane] (Pfaltz & Bauer, Waterbury, CT, USA) overnight in a vacuum desiccator overnight to facilitate detachment of polydimethylsiloxane (PDMS) devices from the master.

5.2.4. PDMS Micromolding. PDMS (Sylgard® 184 Silicone Elastomer Kit, Dow Corning, Midland, MI, USA) was cast against the silicon master to form microfluidic devices. The PDMS prepolymer and crosslinker were mixed at a ratio of 10:1 (w/w) and degassed. For confined migration experiments, an unpatterned, gold-plated wafer was separated from the patterned master by 0.5 mm-thick spacers to create a flat upper surface, and the PDMS was cured in the space between these wafers. Degassed PDMS was poured over the master and cured at 85°C for 2 hours in an oven or on a hot plate. Cured devices were carefully peeled from the mold and diced to size. Inlet ports were created using 4-6 mm-diameter biopsy punches (Harris Uni-Core, Electron Microscopy Sciences, Hatfield, PA, USA). Fabrication of PDMS devices with a free surface, e.g., cured in the absence of the gold wafer (Fig. 5-3A), were slightly curved and caused a
lensing effect that produced aberrations in the lens-free image (Fig. 5-3B). Curing the PDMS device between two silicon wafers (Fig. 5-3C) minimized these aberrations, enabling a clear image of the microchannel array (Fig. 5-3D). Coating one of these wafers with gold decreased the surface forces between the PDMS and the wafer and facilitated removal of the patterned PDMS from the mold.
Figure 5-3. LFI image quality is dependent on the PDMS micromolding technique used. (A) Schematic of typical PDMS micromolding process, in which PDMS is poured over a patterned wafer and cured with a free upper surface exposed to air. Curing PDMS in this manner creates a curved interface at the upper PDMS/air interface, leading to (B) aberrations in the LFI image caused by a lensing effect at the interface. (C) Schematic of micromolding technique in which PDMS is cured between two flat silicon wafers. The upper wafer is coated with gold to facilitate PDMS release. When this method is used, the air-PDMS interface in the imaging path is flat, and (D) a lens effect does not occur, leading to a crisp LFI image. In (C), (D) microchannels are 200 µm long.
5.2.5. Cell culture. MDA-MB-231 or MCF-7 breast adenocarcinoma cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) with 4.5 g/l glucose, L-glutamin, and sodium pyruvate (Corning cellgro, Manassas, VA, USA) supplemented with 10% fetal bovine serum (FBS; Life Technologies Gibco, Carlsbad, CA, USA) and 1% penicillin/streptomycin (P/S; 100 U/ml-100 µg/ml; Gibco) at 37°C and 5% CO₂. Cells were passaged every 3-5 days and were used in experiments between passage 15 and passage 20. Prior to seeding for motility assays, cells were grown to 70-80% confluency, washed with versene (Gibco), detached with 0.05% trypsin-EDTA (Gibco), resuspended in full growth medium, and spun down at 1000 rpm for 5 min. Cells were resuspended in growth medium to a final concentration of 400,000 cells/ml (random motility and microcontact printing assays) or 1x10⁶ cells/ml (wound healing and microchannel assays).

5.2.6. Migration assay preparation. Migration assays were designed to study migration of breast cancer cells on collagen type I in multiple topographic environments. Cells were subjected to varying degrees of topographic confinement to assess the impact of topography on single cell motility. Migration was imaged during a random motility assay (unconfined 2D migration), microcontact printing motility assay (confined 1D migration), and microchannel motility assay (3D confined migration). In all cases, the substrate was first coated with 20 µg/ml collagen type I (BD Biosciences 354236, San Jose, CA) in 0.02 N acetic acid. Pluronic F-127 (Sigma Aldrich P2443, St. Louis, MO, USA) was then applied to block non-specific binding and prevent cell attachment on non-patterned regions. Pluronic forms a stable anti-adhesive coating on cell culture substrates,
repelling cell attachment for ~1 week (Corey et al., 2010; Liu et al., 2002; Kuo et al., 2012).

Specific preparation for each assay is given below. Substrates were produced by gluing glass containment wells to patterned glass slides with PDMS, or by bonding PDMS microfluidic devices to glass slides. The glass slides had a thickness of 700 µm and were patterned with gold markers to facilitate optical focusing of the sample and to serve as landmarks during time lapse imaging. The gold markers were fabricated using a lift-off process, resulting in 50 nm-thick patterned gold regions. To reduce aberration of the holography beam by the meniscus formed at a liquid-air interface, and a glass coverslip was placed on top of the well. Following plating, cells were imaged every 10 min for up to 10 hours using either the phase contrast or LFI imaging setup.

**Random motility assay**

Substrates were washed with ethanol and DI water and dried with filtered air. Substrates were then wetted with a 5% alconox solution and washed in triplicate with DPBS. Collagen type I (20 µg/ml) was added to the chamber to cover the glass base. Collagen absorbed on the glass during an incubation step at 37°C for 1 hr. Following coating, the slide was washed three times with DPBS. The slide was then incubated in a solution of 2% Pluronic-F127 in DI water at room temperature for 1 hr. After a triplicate wash with DPBS, 100 µl of cell solution at 400,000 cells/ml (40,000 cells total) was pipetted over the ECM-coated surface. The reservoir was filled with full growth medium and agitated gently to disperse the cells over the surface. Cells were placed in an incubator at 37°C, 5% CO₂ for 1 hour to attach. Following attachment, the seeding
solution was aspirated, and the slides were washed twice with growth medium to remove non-adherent cells. Sufficient growth medium was then added to fill the containment chambers. Randomly migrating cells were imaged every 10 min for up to 10 hours.

Microcontact printing assay

Substrates were washed with ethanol and DI water and dried with filtered air. PDMS microchannel devices were temporarily bonded to sufficient growth medium was then added to fill the containment chambers. Randomly migrating cells were imaged every 10 min for up to 10 hours. The slides by placing the device, pattern side down, on the slide and applying gentle pressure to create a seal around the microfluidic channels. The device was primed by introducing a 5% alconox solution to the bottom cell inlet well and waiting for the microchannels to fill with fluid. The soap solution was then aspirated, and the channels were rinsed three times with DPBS. Following rinsing, a 20 µg/ml solution of collagen type I in 0.02 N acetic acid was added to all wells of the device to fill the microchannels with collagen. The device was placed in an incubator at 37°C, 5% CO₂ for 1 hour, and collagen adsorbed to the glass slide and the walls of the PDMS device. After incubation, the collagen solution was purged from the channels by triplicate DPBS wash. The PDMS device was carefully peeled from the slide, leaving collagen patterned in a projection of the microchannel design on the glass surface. The slide was washed once more with DPBS, and the containment chamber was filled with 2% Pluronic F127 to prevent cell adhesion to non-patterned regions of the glass. Patterned slides were incubated with Pluronic for 1 hour at room temperature and then washed three times with DPBS prior to the addition of cells. A 100 µl volume of cells at 400,000 cells/ml (40,000 cells µl⁻¹) was
cells total) was pipetted over the patterned region of the coverslip. The containment well was then filled with full growth medium, and the slide was agitated gently to disperse cells over the surface. Cells were placed in an incubator at 37°C, 5% CO₂ for 1-6 hours (1 hour for MDA-MB-231 cells, 3-6 hours for MCF-7 cells) to attach. Following attachment, the seeding solution was aspirated, and the slides were washed twice with growth medium to remove non-adherent cells. Sufficient growth medium was then added to fill the containment chambers. Randomly migrating cells were imaged every 10 min for up to 10 hours.

Microchannel assay

Glass slides and PDMS microchannel devices were washed with ethanol and DI water and dried with filtered air before treatment with oxygen plasma (Harrick PDC-32G plasma cleaner, Harrick Plasma, Ithaca, NY) at 18 W (high) for 2 minutes following a 5 minute chamber evacuation. Activated PDMS devices were bonded to the slides by placing them patterned side down and applying gentle pressure. The devices were coated with 20 µg/ml collagen type I in 0.02 N acetic acid by adding collagen solution to each well of the device and incubating at 37°C, 5% CO₂ for 1 hour. After incubation, the collagen solution was purged from the channels by washing three times with DPBS. The channels were then filled with 2% Pluronic-F127 to prevent non-specific adhesion and incubated for 1 hour at room temperature. Devices were washed in triplicate with DPBS to remove free polymers prior to the addition of cells. A 50 µl volume of cell suspension at 1x10^6 cells/ml (50,000 cells total) was added to the cell inlet port of the device. Cells were allowed to seed at the bases of the microchannels for 5-10 min. Following
aspiration of the cell seeding solution, the device was washed twice to remove non-adherent cells by adding 50 µl of growth medium to all wells of the device. Fresh growth medium (50 µl/well) was added to all wells of the device, which was then covered by a glass coverslip to prevent media evaporation. Cells migrating within the microchannels were imaged every 10 min for up to 10 hours.

5.2.7. **Cell tracking.** Time lapse images were exported as image stacks to ImageJ (NIH, Bethesda, MD, USA). For LFI experiments, phase contrast reconstructions of the holographic images were created at each focal plane for the initial (time=0) image. The optimum focus depth was determined by visual inspection of the cells and LFI reference markers at each depth. Focal depth is a critical parameter for obtaining a high-resolution image. For random motility, microcontact printing, and microchannel migration assays, the centroid of cells was tracked for up to 8 hours using the MTrackJ plugin (Meijering et al., 2012). Cells that were elongated on printed lines or fully within microchannels for less than 2 hours were not tracked. Tracks were discontinued if cells exited the microchannels or printed regions. Dividing cells were not tracked.

Calculations were made based on two-hour cell migration trajectories. Time and coordinate data were used to calculate the average instantaneous cell speed for each cell, defined as the average of point-to-point (10 min) speeds for a given cell over the course of tracking. The persistence ratio of migration was calculated as the net displacement of the cell over the course of tracking divided by the total distance traveled by the cell. Cell velocity was calculated as the displacement of the cell divided by the tracking time. Mean squared displacement (MSD), primary persistence, and total diffusivity were calculated
using the APRW model as described previously (Wu et al., 2014; Wu et al., 2015), with the maximum persistence time constrained to 1000 min.

Additionally, cell speed, velocity, and persistence ratio were calculated for trajectories of varying tracking time (2-8 hours), as described previously (Hung et al., 2013; Irimia et al., 2009; Tong et al., 2012). Average tracking times between phase and LFI experiments were identical in this scenario. For these trajectories, APRW parameters were not calculated because MSD data became weighted as fewer cells were tracked for longer periods (cells could enter and leave microchannels and microcontact printed surfaces prior to 8 hours of tracking). The exception is the random motility data, where all cells were tracked for 8 hours, and APRW parameters could be calculated for an 8 hour experiment time.

5.2.8. Statistics. For each experiment, 30 cell tracks were randomly chosen, and each experiment was run in three biologically independent repeats, giving a total of 90 cells per condition. There were two exceptions to this experimental design: (1) MCF7 microcontact printing experiments, where 30 cell tracks from two biologically independent experiments were grouped to give a total of 60 cell trajectories per condition; (2) MCF-7 microchannel experiments, where 25 cell tracks from three biologically independent experiments were grouped to give a total of 75 cell trajectories per condition. These exceptions were made because MCF-7 cells were less readily able to spread on confining, 6 µm-wide printed lines or enter microchannels. Experimental design was performed in this manner so that no single biological repeat would weigh the overall results if a large or small number of cells entered the microchannels or adhered to
the printed lines. Results for a given cell type that were generated using LFI or phase contrast microscopy were compared using an unpaired t test or Mann-Whitney test after assessing the normality of the data via a D’Agostino & Pearson omnibus normality test. If populations were normally distributed but had significantly different variances, an unpaired t test with Welch’s correction was used. Results across cell types were compared using a Kruskal-Wallis test with Dunn’s multiple comparisons post-test.

5.3. Results

5.3.1. Compact lens-free imaging platform produces large field-of-view images in which cell morphology is clearly visible. The LFI technique is based on the principle of in-line digital holography. A schematic representation of the principle and picture of the setup is shown in Figures 5-1 and 5-2. In-line holography was chosen for cell migration assays because the imaged objects are transparent and the more complex optics needed to maintain a separate reference beam can be avoided. The LFI was assembled and operated in a standard cell culture incubator (37°C, 5% CO₂; Fig. 5-2), circumventing the cost of custom-built stage-top environments.

The LFI technique produced images with a field-of-view (FOV) of 6.4 mm x 4.6 mm in which morphology at the single-cell level was clearly visible (Fig. 5-1). A single LFI image covered ~29,400,000 µm², an approximately 50-fold increase in imaged area compared to a phase contrast image taken with a 10x objective (~606,000 µm² imaged area). LFI images provided detail of cell morphology similar to that obtained with phase contrast imaging, with a resolution of 1.3 µm (Fig. 5-1). The impressive field-of-view and optical range allow for elimination of automated microscope stage translation without sacrificing information about cell morphology. Importantly, FOV and resolution...
of LFI are provisionally limited by chip and pixel size, respectively. Additionally, focusing is achieved through variation of a digital parameter, such that one image contains 3-dimensional (3D) information, and stage drift can be digitally corrected. Stage drift is a common problem in time lapse imaging using stage-top incubators (Frigault et al., 2009; Paddock, 2001; Stephens et al., 2003; Adler et al., 2003). Furthermore, time lapse LFI did not affect cell viability (data not shown). These advantageous features avert both the cost and the problems of programmable, motorized, climate-controlled microscopy stages. Hence, as laboratories could purchase 50 LFI systems for the price of one conventional imaging tool, live cell research could advance at a much faster pace.

5.3.2. **Lens-free and phase contrast imaging produce quantitatively similar results for a wide range of single-cell migration assays.** To benchmark LFI for use in single-cell migration assays spanning a range of physiologically relevant microenvironments, we imaged MDA-MB-231 and MCF7 breast carcinoma cells during random migration and confined migration on microcontact lines and within microchannels. For a given experiment, a flask of cells was split, with a portion of the population imaged on a phase contrast microscope while another subpopulation was simultaneously imaged via LFI. All substrates were coated with 20 μg/ml collagen type I. In microcontact printing assays, 6 μm-wide, 600 μm-long lines of collagen type I were surrounded by non-adhesive regions coated with Pluronic F127. In microchannel assays, cells migrated inside 6 μm-wide, 10-μm tall, 600 μm-wide microchannels. Examples of the FOVs captured from single LFI and phase contrast images for each assay are shown in Figure 5-4.
**Figure 5-4.** Representative images from the cell migration assays illustrate the large field of view of the LFI platform. Unprocessed, unzoomed images from each assay for the phase contrast (10x objective) and LFI imaging platforms are shown. LFI offers a significantly larger field of view for a single image. Assays shown are (A) random motility assay, (B) microcontact printing migration assay, and (C) microchannel migration assay, all performed with MDA-MB-231 cells. In phase contrast images, scale bar represents 50 µm. In LFI images, scale bar represents 1 mm.
For a given migration assay and cell type, quantitative descriptors of cell migration were calculated from cell trajectories captured using either the phase contrast or LFI platform. Measurements were made to reflect both point-to-point (speed, persistence) and endpoint (velocity) descriptors of cell migration. Additionally, trajectory data was fit to the recently described anisotropic random walk model (Wu et al., 2014; Wu et al., 2015) in each microenvironment. Results are summarized in Figures 5-5, 5-6, and 5-7. For a given cell type and metric, results generated using the imaging systems were similar (and typically not significantly different by statistical tests), and cell morphology was clear in each microenvironment. Mean squared displacements (MSDs) calculated from trajectories obtained using either LFI or phase contrast imaging were also similar. We noted that LFI generated faster migration quantifiers (speed, velocity, and total diffusivity) than phase contrast imaging for MCF7 cells migrating within microchannels (Fig. 5-7D,E,G). However, given that results for MCF7 cells in other microenvironments were not dependent on the imaging platform, and that phase contrast and LFI microchannel data were indistinguishable for MDA-MB-231 cells, we concluded that faster migration is not a characteristic of cells imaged using LFI.
Figure 5-5. Phase contrast and LFI imaging platforms generate similar results for random motility assays. Time lapse images of (A) MDA-MB-231 and (B) MCF7 breast adenocarcinoma cells migrating on collagen type I-coated glass slides and imaged using either phase contrast microscopy (10x objective) or the LFI platform. Scale bars represent 50 µm. (C) Mean squared displacements observed for the two cell types with each imaging platform. (D) Average migration speed, (E) velocity, (F) persistence ratio, (G) total diffusivity, and (H) primary persistence time for MDA-MB-231 and MCF7 cells following analysis of cell trajectories obtained using either
phase contrast imaging or LFI. For all metrics, cell trajectories were analyzed over 2 hours. N=90 cells/condition, with 30 cells/experiment analyzed over 3 independent experiments. Statistical significance between phase contrast and LFI imaging results was analyzed by an unpaired t test if cells passed the D’Agostino and Pearson omnibus normality test, or by Mann-Whitney test if they did not. Differences between MDA-MB-231 and MCF7 cells were assessed by Kruskal-Wallis test with Dunn’s multiple comparisons post-test. n.s., difference not statistically significant; *, p<0.05; ****, p<0.0001.
Figure 5-6. Phase contrast and LFI imaging platforms generate similar results for microcontact printing migration assays. Time lapse images of (A) MDA-MB-231 and (B) MCF7 breast adenocarcinoma cells migrating on 6 µm-wide collagen type I printed lines and imaged using either phase contrast microscopy (10x objective) or the LFI platform. Scale bars represent 50 µm. (C) Mean squared displacements observed for the two cell types with each imaging platform. (D) Average migration speed, (E) velocity, (F) persistence ratio, (G) total diffusivity, and (H) primary persistence time for MDA-MB-231 and MCF7 cells following
analysis of cell trajectories obtained using either phase contrast imaging or LFI. For all metrics, cell trajectories were analyzed over 2 hours. For MDA-MB-231 cells, N=90 cells/condition, with 30 cells/experiment analyzed over 3 independent experiments. For MCF7 cells, N=60 cells/condition, with 30 cells/experiment analyzed over 2 independent experiments. Statistical significance between phase contrast and LFI imaging results was analyzed by Mann-Whitney test. Differences between MDA-MB-231 and MCF7 cells were assessed by Kruskal-Wallis test with Dunn’s multiple comparisons post-test. n.s., difference not statistically significant; *, p<0.05; ****, p<0.0001.
Figure 5-7. Phase contrast and LFI imaging platforms generate similar results for microchannel migration assays. Time lapse images of (A) MDA-MB-231 and (B) MCF7 breast adenocarcinoma cells migrating through 6 µm-wide, 10 µm-tall collagen type I-coated PDMS microchannels and imaged using either phase contrast microscopy (10x objective) or the LFI platform. (C) Mean squared displacements observed for the two cell types with each imaging platform. (D) Average migration speed, (E) velocity, (F) persistence ratio, (G) total diffusivity, and (H) primary persistence time for MDA-MB-231 and MCF7 cells following analysis of cell
trajectories obtained using either phase contrast imaging or LFI. For all metrics, cell trajectories were analyzed over 2 hours. For MDA-MB-231 cells, N=90 cells/condition, with 30 cells/experiment analyzed over 3 independent experiments. For MCF7 cells, N=75 cells/condition, with 25 cells/experiment analyzed over 3 independent experiments. Statistical significance between phase contrast and LFI imaging results was analyzed by Mann-Whitney test. Differences between MDA-MB-231 and MCF7 cells were assessed by Kruskal-Wallis test with Dunn’s multiple comparisons post-test. n.s., difference not statistically significant; *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001.
Of note, similar results were generated when cells were tracked for 8 hours during random migration, or for times between 2-8 hours during confined migration (dependent on the time cells spent inside microchannels or on microcontact printed lines), as described previously (Irimia et al., 2009) (Figs. 5-8, 5-9, 5-10). Tracking time had little effect on measured cell speed and no significant effect on total diffusivity, though persistence ratios were typically higher for cells tracked for 2 h in comparison to those tracked for longer time periods (Figs. 5-11, 5-12, 5-13). However, there were no significant differences in primary persistence times calculated using the APRW model.
Figure 5. Phase contrast and LFI imaging platforms generate similar results for random motility assays over longer (8 h) imaging times. Time lapse images of (A) MDA-MB-231 and (B) MCF7 breast adenocarcinoma cells migrating on collagen type I-coated glass slides and imaged using either phase contrast microscopy (10x objective) or the LFI platform. Scale bars represent 50 µm. (C) Mean-squared displacements observed for the two cell types with each imaging platform. (D) Average migration speed, (E) velocity, (F) persistence ratio, (G) total
diffusivity, and (H) primary persistence time for MDA-MB-231 and MCF7 cells following analysis of cell trajectories obtained using either phase contrast imaging or LFI. For all metrics, cell trajectories were analyzed over 8 hours. N=90 cells/condition, with 30 cells/experiment analyzed over 3 independent experiments. Statistical significance between phase contrast and LFI imaging results was analyzed by an unpaired t test if cells passed the D’Agostino and Pearson omnibus normality test, or by Mann-Whitney test if they did not. Differences between MDA-MB-231 and MCF7 cells were assessed by Kruskal-Wallis test with Dunn’s multiple comparisons post-test. n.s., difference not statistically significant; **, p<0.01; ****, p<0.0001.
Figure 5-9. Phase contrast and LFI imaging platforms generate similar results for microcontact printing migration assays over longer imaging times. Time lapse images of (A) MDA-MB-231 and (B) MCF7 breast adenocarcinoma cells migrating on 6 µm-wide collagen type I printed lines and imaged using either phase contrast microscopy (10x objective) or the LFI platform. Scale bars represent 50 µm. (C) Tracking time, (D) average migration speed, (E) velocity, and (F) persistence ratio for MDA-MB-231 and MCF7 cells following analysis of cell trajectories obtained using either phase contrast imaging or LFI. Cell trajectories were analyzed over 2-8 hours, depending on the length of time cells remained on the printed lines. For MDA-MB-231 cells, N=90 cells/condition, with 30 cells/experiment analyzed over 3 independent experiments. For MCF7 cells, N=60 cells/condition, with 30 cells/experiment analyzed over 2 independent experiments. Statistical significance between phase contrast and LFI imaging results was analyzed by an unpaired t test if cells passed the D’Agostino and Pearson omnibus normality test, or by Mann-Whitney test if they did not. Differences between MDA-MB-231 and MCF7
cells were assessed by Kruskal-Wallis test with Dunn’s multiple comparisons post-test. n.s.,
difference not statistically significant; *, p<0.05; **, p<0.01; ****, p<0.0001.
Figure 5-10. Phase contrast and LFI imaging platforms generate similar results for microchannel migration assays over longer imaging times. Time lapse images of (A) MDA-MB-231 and (B) MCF7 breast adenocarcinoma cells migrating through 6 µm-wide, 10 µm-tall collagen type I-coated PDMS microchannels and imaged using either phase contrast microscopy (10x objective) or the LFI platform. (C) Tracking time, (D) average migration speed, (E) velocity, and (F) persistence ratio for MDA-MB-231 and MCF7 cells following analysis of cell trajectories obtained using either phase contrast imaging or LFI. Cell trajectories were analyzed over 2-8 hours, depending on the length of time cells remained in the microchannels. For MDA-MB-231 cells, N=90 cells/condition, with 30 cells/experiment analyzed over 3 independent experiments. For MCF7 cells, N=75 cells/condition, with 25 cells/experiment analyzed over 3 independent experiments. Statistical significance between phase contrast and LFI imaging results was analyzed by Mann-Whitney. Differences between MDA-MB-231 and MCF7 cells were
assessed by Kruskal-Wallis test with Dunn's multiple comparisons post-test. n.s., difference not statistically significant; *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001.
Figure 5-11. Quantitative descriptors of random MDA-MB-231 and MCF7 cell motility are dependent on the tracking time used during the experiment. For random motility assays, MDA-
MB-231 and MCF7 breast adenocarcinoma cell trajectories were analyzed over either 2 h (Fig. 3) or 8 h (Supplementary Fig. 4). Results generated by a given imaging platform (phase contrast or LFI) for each analysis time frame were compared. Metrics analyzed were: (A,B) average speed, (C,D) velocity, (E,F) persistence ratio, (G,H) total diffusivity, and (I,J) primary persistence time. For random motility assays, we noted little change in speed as tracking time increased, although persistence ratio consistently decreased with longer tracking times. Differences in the primary persistence time were not significant, but there was a clear trend toward shorter persistence times as the time that the cell trajectory was analyzed increased. N=90 cells/condition, with 30 cells/experiment analyzed over 3 independent experiments. Statistical significance between 2 h results and 8 h results were assessed via unpaired t-test with Welch’s correction if cells passed the D’Agostino and Pearson omnibus normality test, or by Mann-Whitney test if they did not. n.s., difference not statistically significant; *, p<0.05; **, p<0.01; ****, p<0.0001.
Figure 5-12. Velocity and persistence but not speed are dependent on the tracking time used during migration of MDA-MB-231 and MCF7 cells on microcontact printed lines. For microcontact printing migration assays, MDA-MB-231 and MCF7 breast adenocarcinoma cell trajectories were analyzed over either 2 h (Fig. 4) or for variable times between 2-8 hours, depending on the amount of time the cell spent on the printed line (Supplementary Fig. 5). Results generated by a given imaging platform (phase contrast or LFI) for each analysis time frame were compared. Metrics analyzed were: (A,B) average speed, (C,D) velocity, and (E,F) persistence ratio. For microcontact printing migration assays, we noted little change in speed as tracking time increased, although persistence ratio consistently decreased with longer tracking times. For MDA-MB-231 cells, N=90 cells/condition, with 30 cells/experiment analyzed over 3
independent experiments. For MCF7 cells, N=60 cells/condition, with 30 cells/experiment analyzed over 2 independent experiments. Statistical significance between 2 h results and variable tracking time results were assessed via unpaired t-test or unpaired t-test with Welch’s correction (for populations with significantly different variances) if cells passed the D’Agostino and Pearson omnibus normality test, or by Mann-Whitney test if they did not. n.s., difference not statistically significant; *, p<0.05; **, p<0.01; ***, p<0.001.
Figure 5-13. Quantitative descriptors of MDA-MB-231 and MCF7 cell migration in confining microchannels display different dependence on tracking time. For microchannel migration assays, MDA-MB-231 and MCF7 breast adenocarcinoma cell trajectories were analyzed over either 2 h (Fig. 5) or for variable times between 2-8 hours, depending on the amount of time the cell spent on the printed line (Supplementary Fig. 6). Results generated by a given imaging platform (phase contrast or LFI) for each analysis time frame were compared. Metrics analyzed were: (A,B) average speed, (C,D) velocity, and (E,F) persistence ratio. For microchannel migration assays, we noted little change in speed as tracking time increased, although persistence ratio consistently decreased with longer tracking times for MCF7 cells. For MDA-MB-231 cells, N=90 cells/condition, with 30 cells/experiment analyzed over 3 independent experiments.
MCF7 cells, N=75 cells/condition, with 25 cells/experiment analyzed over 3 independent experiments. Statistical significance between 2 h results and variable tracking time results were assessed via unpaired t-test if cells passed the D’Agostino and Pearson omnibus normality test, or by Mann-Whitney test if they did not. n.s., difference not statistically significant; **, p<0.01; ***, p<0.001; ****, p<0.0001.
5.4. Discussion

Issues with time-lapse imaging involve maintenance of suitable environments for both the organisms being imaged and the hardware used to capture these images. Cells must be maintained in an environment with a controlled temperature and CO\textsubscript{2} concentration, and phototoxicity and media evaporation must be minimized (Coutu et al., 2013; Frigault et al., 2009; Stephens et al., 2003). For this purpose, laboratories acquire expensive, custom-built stage top incubators that are designed to fit the specific optical tool and experimental conditions desired. Common problems with this approach are: damage to microscope hardware by the humidity in the chamber necessary to avoid evaporation and changes in osmolarity (Frigault et al., 2009); limited temporal resolution due to mechanical parts, e.g. motorized microscope stage translation (Coutu et al., 2013); long times necessary to stabilize enclosure temperatures (Frigault et al., 2009); stage drift caused by changes in room temperature (Frigault et al., 2009; Paddock, 2001; Stephens et al., 2003) or heating of microscope components (Adler et al., 2003); and inflexible setups with optical tables overflowing with cables and tubes to component controllers, CO\textsubscript{2} tanks, light sources, etc. Complex autofocus routines can help with stage drift, but they require additional illumination of the sample (Stephens et al., 2003). The need for specialized microscopes, with expensive features and custom-built cell culture environments, hinders live cell research, as the number of experiments being done is limited by the number of imaging tools available to the laboratory.

Here, a variety of cell migration assays spanning multiple dimensions and migration spaces on the order of those found \textit{in vivo} (Wolf et al., 2009; Weigelin et al., 2012) were used to establish LFI as a viable imaging technique for \textit{in vitro} cell migration
assays. Comparison of experimental results obtained using the LFI setup with those from a conventional phase contrast microscope validates this new time-lapse imaging technique. Importantly, the LFI technique generated these results by imaging a 50-fold larger area with 50-fold less expensive equipment, and this equipment was housed in a conventional cell culture incubator common to cell biology laboratories.

Interestingly, while metastatic MDA-MB-231 cells displayed significantly higher average speeds, velocities, and diffusivities than MCF7 cells during random migration, there was no significant difference in the persistence ratio between these cell types in 2D, or between the primary persistence calculated from the APRW model (Figs. 5-4, 5-7). In contrast, microcontact printing and microchannel migration assays were able to distinguish between MDA-MB-231 and MCF7 cells for all metrics analyzed (Fig. 5-5, 5-6, 5-8, 5-9). These results suggest that more complex microenvironments that better recapitulate in vivo migration spaces are better able to distinguish between metastatic and non-metastatic cell lines. Microenvironmental regulation of migration is explored in greater detail in the following chapters.
Chapter 6

PHYSICAL CONFINEMENT DRIVES RAPID AND PERSISTENT MIGRATION OF MDA-MB-231 AND MCF7 CELLS

6.1. Introduction

The high-throughput, time lapse nature of LFI, established in the previous chapter, enabled us to study how metastatic MDA-MB-231 and non-metastatic MCF7 cells react to different forms of physical confinement. Analysis of cell migration on 2D surfaces, on 1D microcontact printed lines, and in 3D microchannels revealed that these cells migrate more efficiently upon increasing confinement. Specifically, we demonstrated that lateral 1D confinement on printed lines and 3D confinement within microchannels drive differential cell migration responses. Additionally, point-to-point cell location measurements (instead of endpoint measurements) elucidated the mechanisms of motility in each microenvironment. The coefficient of variation in speed of a given MDA-MB-231 cell was inversely correlated to the average speed of that cell, demonstrating how highly invasive cells may explore their environment not only by moving at high average speeds but also by migrating with little change in speed over time. This work provides important information on microenvironmental regulation of cell behavior.
6.2. Materials and Methods

6.2.1. Cell culture, imaging methods, and assay preparation. Cell culture was performed exactly as described in Chapter 5, as were imaging (LFI and phase contrast) and cell migration assays.

6.2.2. Cell tracking. Time lapse images were exported as image stacks to ImageJ (NIH, Bethesda, MD, USA). For LFI experiments, phase contrast reconstructions of the holographic images were created at each focal plane for the initial (time=0) image. The optimum focus depth was determined by visual inspection of the cells and LFI reference markers at each depth. For random motility, microcontact printing, and microchannel migration assays, the centroid of cells was tracked for up to 8 hours using the MTrackJ plugin (Meijering et al., 2012). Cells that were elongated on printed lines or fully within microchannels for less than 2 hours were not tracked. Tracks were discontinued if cells exited the microchannels or printed regions. Dividing cells were not tracked.

Calculations were made based on two-hour cell migration trajectories. Time and coordinate data were used to calculate the average instantaneous cell speed for each cell, defined as the average of point-to-point (10 min) speeds for a given cell over the course of tracking. The persistence ratio of migration was calculated as the net displacement of the cell over the course of tracking divided by the total distance traveled by the cell. Cell velocity was calculated as the displacement of the cell divided by the tracking time. Mean squared displacement (MSD), primary persistence, and total diffusivity were calculated using the APRW model as described previously (Wu et al., 2014; Wu et al., 2015), with the maximum persistence time constrained to 1000 min.
Additionally, cell speed, velocity, and persistence ratio were calculated for trajectories of varying tracking time (2-8 hours), as described previously (Hung et al., 2013; Irimia et al., 2009; Tong et al., 2012). Average tracking times between phase and LFI experiments were identical in this scenario. For these trajectories, APRW parameters were not calculated because MSD data became weighted as fewer cells were tracked for longer periods (cells could enter and leave microchannels and microcontact printed surfaces prior to 8 hours of tracking). The exception was the random motility data, where all cells were tracked for 8 hours, and APRW parameters could be calculated for an 8 hour experiment time.

6.2.3. Statistics. For each experiment, 30 cell tracks were randomly chosen, and each experiment was run in three biologically independent repeats, giving a total of 90 cells per condition. There were two exceptions to this experimental design: (1) MCF7 microcontact printing experiments, where 30 cell tracks from two biologically independent experiments were grouped to give a total of 60 cell trajectories per condition; (2) MCF-7 microchannel experiments, where 25 cell tracks from three biologically independent experiments were grouped to give a total of 75 cell trajectories per condition. These exceptions were made because MCF-7 cells were less readily able to spread on confining, 6 µm-wide printed lines or enter microchannels. Experimental design was performed in this manner so that no single biological repeat would weigh the overall results if a large or small number of cells entered the microchannels or adhered to the printed lines.
Results across microenvironments were compared using a Kruskal-Wallis test with Dunn’s multiple comparisons post-test. Correlation coefficients between speed and variance in speed in different microenvironments were assessed using Spearman’s correlation coefficient. All statistical calculations were performed in GraphPad Prism 6 software.

6.3. Results

6.3.1. 1D vs. 3D physical confinement differentially regulates cell motility. To test how different migration microenvironments affected cell migration, we asked whether 1D confinement on microcontact printed lines and 3D confinement within microchannels produced identical migration results. Interestingly, we found that both types of confinement increased migration speed, velocity, and persistence compared to random 2D migration for MDA-MB-231 and, to a lesser extent, MCF7 cells (Fig. 6-1). However, these metrics tended to be significantly higher in microchannels compared to on microcontact printed surfaces, suggesting that migration was most efficient when cells were confined in 3D microchannels (Fig. 6-1). Increasing migration efficiency upon increasing physical confinement was demonstrated by plots of MSDs, which demonstrate larger average displacements as cells were increasingly confined to printed lines and further to 3D microchannels (Fig. 6-1). Similar results were obtained when phase contrast imaging was used (Fig. 6-2), or when cells imaged using LFI or phase contrast imaging were tracked for longer times (Fig. 6-3). These results suggest that careful consideration of the microenvironment is required when designing experiments to test the effects of physical cues on migration, as not all types of physical confinement result in functionally identical results.
Figure 6-1. Increasing confinement results in more efficient migration of MDA-MB-231 and MCF7 cells (LFI imaging). Results generated via LFI imaging across differing levels of physical cell confinement were compared. Mean squared displacements for (A) MDA-MB-231 and (B) MCF7 cells in different physical microenvironments. (C) Average speed, (D) velocity, (E) persistence ratio, (F) total diffusivity, and (G) primary persistence time for MDA-MB-231 and MCF7 cells in each microenvironment. Plots were generated from LFI cell trajectories, with cells tracked for 120 min. For MDA-MB-231 cells, N=90 cells/condition, with 30 cells/experiment.
analyzed over 3 independent experiments. For MCF7 cells: N=90 cells, with 30 cells/experiment analyzed over 3 independent experiments for 2D results; N=60 cells, with 30 cells/experiment analyzed over 2 independent experiments for printed lines; and N=75 cells, with 25 cells/experiment analyzed over 3 independent experiments for microchannel results. Comparisons between microenvironments for a given cell type were made with Kruskal-Wallis test with Dunn’s multiple comparisons post-test. n.s., difference not statistically significant; **, p<0.01; ***, p<0.001; ****, p<0.0001.
Figure 6-2. Increasing confinement results in more efficient migration of MDA-MB-231 and MCF7 cells (phase contrast imaging). Results generated via phase contrast imaging across differing levels of physical cell confinement were compared. Mean squared displacements for (A) MDA-MB-231 and (B) MCF7 cells in different physical microenvironments. (C) Average speed, (D) velocity, (E) persistence ratio, (F) total diffusivity, and (G) primary persistence time for MDA-MB-231 and MCF7 cells in each microenvironment. Plots were generated from phase contrast cell trajectories, with cells tracked for 120 min. For MDA-MB-231 cells, N=90 cells/condition, with 30 cells/experiment analyzed over 3 independent experiments. For MCF7
cells: N=90 cells, with 30 cells/experiment analyzed over 3 independent experiments for 2D results; N=60 cells, with 30 cells/experiment analyzed over 2 independent experiments for printed lines; and N=75 cells, with 25 cells/experiment analyzed over 3 independent experiments for microchannel results. Comparisons between microenvironments for a given cell type were made with Kruskal-Wallis test with Dunn’s multiple comparisons post-test. n.s., difference not statistically significant; *, p<0.05; **, p<0.01; ****, p<0.0001.
Figure 6-3. Increasing confinement results in more efficient cell migration of MDA-MB-231 and MCF7 cells, regardless of cell tracking time. Results generated via (A-C) LFI imaging and (D-F) phase contrast imaging across differing levels of physical cell confinement were compared for cell tracking times between 2-8 h. (A,D) Average speed, (B,E) velocity, and (C,F) persistence ratio for MDA-MB-231 and MCF7 cells in each microenvironment. Plots were generated from cell trajectories lasting from 2-8 h, dependent on the amount of time cells remained on microcontact printed lines or within microchannels. Trajectories of cells migrating in 2D microenvironments were analyzed for 8 h. For MDA-MB-231 cells, N=90 cells/condition, with 30 cells/experiment analyzed over 3 independent experiments. For MCF7 cells: N=90 cells, with 30 cells/experiment analyzed over 3 independent experiments for 2D results; N=60 cells, with 30
cells/experiment analyzed over 2 independent experiments for printed lines; and N=75 cells, with 25 cells/experiment analyzed over 3 independent experiments for microchannel results. Comparisons between microenvironments for a given cell type were made with Kruskal-Wallis test with Dunn’s multiple comparisons post-test. n.s., difference not statistically significant; **, p<0.01; ***, p<0.001; ****, p<0.0001.
6.3.2. Microenvironmental exploration by metastatic tumor cells is aided by rapid migration with minimal speed variance. To demonstrate the utility of time lapse (as opposed to endpoint) assays in understanding cell migration, we asked how the coefficient of variation in speed of a single cell correlated with the average speed of that cell over the course of tracking. Consistently and across migration assays, we found that MDA-MB-231 cells migrating with the highest average speeds had the smallest coefficients of variation in speed, indicating that these cells moved very quickly over the entire time course of migration (Fig. 6-4). There was a statistically significant correlation between these metrics in random motility and microchannel assays imaged using the LFI platform (Fig. 6-4), and the correlation was significant in all three microenvironments when phase contrast imaging was used (Fig. 6-5). In general, we did not see these correlations in MCF7 cells, where the coefficient of variation in speed was not statistically correlated to the average speed (Figs. 6-4, 6-5). Similar results were obtained when longer tracking times were used (Fig. 6-6). These results suggest that the most aggressive tumor cells explore their environment not only by moving at high speeds but by continually moving at high speeds. Indeed, both displacement and total diffusivity, two indicators of the extent of environmental exploration, were positively correlated with cell speed for MDA-MB-231 cells in all microenvironments tested (Fig. 6-7). The strongest correlations were seen in microchannels, where topological features potentiated rapid movement away from the initial cell position. This may be especially relevant in the context of cancer metastasis, where collagen fibers aligned perpendicularly to the tumor boundary are associated with breast cancer cell invasion (Provenzano et al., 2006) and a poor prognosis (Conklin et al., 2011).
Figure 6-4. The fastest MDA-MB-231 cells migrate with the smallest coefficient of variation in speed, regardless of the migration microenvironment, in cells imaged with the LFI platform.

The coefficient of variation in speed for a given cell was plotted against the average speed of that cell over a 2 h tracking time for both (A-C) MDA-MB-231 and (D-F) MCF7 breast adenocarcinoma cells. Plots were made for cells migrating (A,D) on 2D surfaces, (B,E) on 6 µm-wide microcontact printed lines, and (C,F) in 6 µm-wide, 10 µm-tall microchannels. Cells were imaged using LFI. The variance in speed of MDA-MB-231 cells was inversely correlated to average cell speed in all microenvironments tested, while the variance in speed of MCF7 cells showed little relationship to cell speed. Spearman correlation coefficients (r) were computed, as were the two-tailed p values for each correlation. For MDA-MB-231 cells, N=90 cells/condition, with 30 cells/experiment analyzed over 3 independent experiments. For MCF7 cells: N=90 cells, with 30 cells/experiment analyzed over 3 independent experiments for 2D results; N=60 cells, with 30 cells/experiment analyzed over 2 independent experiments for printed lines; and N=75 cells, with 25 cells/experiment analyzed over 3 independent experiments for microchannel results.
Figure 6-5. The fastest MDA-MB-231 cells migrate with the smallest coefficient of variation in speed, regardless of the migration microenvironment, in cells imaged with phase contrast microscopy. The coefficient of variation in speed for a given cell was plotted against the average speed of that cell over a 2 h tracking time for both (A-C) MDA-MB-231 and (D-F) MCF7 breast adenocarcinoma cells. Plots were made for cells migrating (A,D) on 2D surfaces, (B,E) on 6 µm-wide microcontact printed lines, and (C,F) in 6 µm-wide, 10 µm-tall microchannels. Cells were imaged using phase contrast microscopy. The variance in speed of MDA-MB-231 cells was inversely correlated to average cell speed in all microenvironments tested, while the variance in speed of MCF7 cells showed no significant relationship to cell speed. Spearman correlation coefficients (r) were computed, as were the two-tailed p values for each correlation. For MDA-MB-231 cells, N=90 cells/condition, with 30 cells/experiment analyzed over 3 independent experiments. For MCF7 cells: N=90 cells, with 30 cells/experiment analyzed over 3 independent experiments for 2D results; N=60 cells, with 30 cells/experiment analyzed over 2 independent experiments for printed lines; and N=75 cells, with 25 cells/experiment analyzed over 3 independent experiments for microchannel results.
Figure 6-6. The fastest MDA-MB-231 cells migrate with the smallest coefficient of variation in speed, regardless of the migration microenvironment, for longer cell migration periods. The coefficient of variation in speed for a given cell was plotted against the average speed of that cell over a 2-8 h tracking time for both (A-C, G-I) MDA-MB-231 and (D-F, G-J) MCF7 breast adenocarcinoma cells. Plots were made for cells migrating (A,D,G,J) on 2D surfaces, (B,E,H,K)
on 6 µm-wide microcontact printed lines, and \((\text{C,F,I,L})\) in 6 µm-wide, 10 µm-tall microchannels. Cells were imaged using \((\text{A-F})\) LFI or \((\text{G-L})\) phase contrast microscopy. Plots were generated from cell trajectories lasting from 2-8 h, dependent on the amount of time cells remained on microcontact printed lines or within microchannels. Trajectories of cells migrating in 2D microenvironments were analyzed for 8 h. The variance in speed of MDA-MB-231 cells was inversely correlated to average cell speed in all microenvironments tested, while the variance in speed of MCF7 cells showed little significant relationship to cell speed. Spearman correlation coefficients \((r)\) were computed, as were the two-tailed \(p\) values for each correlation. For MDA-MB-231 cells, \(N=90\) cells/condition, with 30 cells/experiment analyzed over 3 independent experiments. For MCF7 cells: \(N=90\) cells, with 30 cells/experiment analyzed over 3 independent experiments for 2D results; \(N=60\) cells, with 30 cells/experiment analyzed over 2 independent experiments for printed lines; and \(N=75\) cells, with 25 cells/experiment analyzed over 3 independent experiments for microchannel results.
Fig. 6-7. Faster MDA-MB-231 cells explore more of their microenvironment, with confinement enhancing displacement from initial cell position. The (A-C) displacement and (D-E) total diffusivity for a given cell (imaged using LFI over a 2 h tracking period) was plotted against the average speed of that cell for MDA-MB-231 cells migrating in (A,D) 2D microenvironments, (B,E) on 6 µm-wide microcontact printed lines, and (C,F) in 6 µm-wide, 10 µm-tall microchannels. Spearman correlation coefficients (r) were computed, as were the two-tailed p values for each correlation. The displacement and total diffusivity were correlated to the average cell speed in all microenvironments, with the strongest correlations seen in microchannels. N=90 cells/condition, with 30 cells/experiment analyzed over 3 independent experiments.
6.4. Discussion

The high-throughput nature of LFI experiments allowed assessment of how different migration microenvironments affected the migration behavior of MDA-MB-231 and MCF7 breast adenocarcinoma cells. Physical confinement led to more efficient migration than that seen on 2D surfaces. However, lateral 1D confinement and 3D confinement within microchannels drove differential cell migration responses. Cells in microchannels were more persistent and faster moving than cells on printed lines, illustrating the importance of 3D topology in modulating cell migration.

These experiments provide an important proof of concept for the utility of the LFI platform. By increasing the throughput and decreasing the cost of time lapse in vitro experiments, LFI may enable discovery of additional fundamental migration mechanisms. Random cell migration assays on glass (Kim et al., 2013), plastic (Agus et al., 2013), or hydrogel surfaces (Stroka et al., 2009; Pushkarsky et al., 2014; Kim et al., 2013) are easily performed in cell biology labs and require no specialized equipment for substrate preparation. Random migration assays may allow high-throughput screening of potential pathways involved in breast cancer metastasis (van Roosmalen et al., 2015). Cell migration on printed microchannel lines has demonstrated a correlation between cell speed and persistence (Maiuri et al., 2012) that has generated significant information on mechanisms of cell motility (Maiuri et al., 2015), and some research groups use it as an analog for 3D migration (Doyle et al., 2009; Sharma et al., 2012).

That said, there are important differences between 2D microenvironments imaged in previous LFI studies (Pushkarsky et al., 2014) and the physiological microenvironment. While 2D assays are readily transferable to LFI imaging, they do not
reflect differences between metastatic and non-metastatic cell lines as readily as 3D confined migration assays (see Chapter 6). Cells in the body migrate through protein-free migration tracks with cross-sectional areas of ~10-1000 \( \text{um}^2 \) pore area (Wolf et al., 2009), often without degrading the microenvironment (Weigelin et al., 2012), and aspects of these migration tracks are recapitulated by microchannel migration devices. Importantly, microchannel assays have repeatedly elucidated cell migration behavior not predicted by 2D assays (Hung et al., 2013; Stroka et al., 2014b; Balzer et al., 2012). Microchannel microfluidic devices in combination with phase contrast imaging can be used for high throughput drug screening or to study the effect of EMT on cells (Zhang et al., 2014), or to examine hypoxia and primary cell populations (Zhang et al., 2015). Furthermore, microfluidic point-of-care assays reliant on migration through PDMS microchannels are already in development to study the immune response from whole-blood samples (Boneschansker et al., 2014; Hamza et al., 2015; Jones et al., 2014). The results presented here demonstrate that LFI that could make physiologically-relevant microchannel migration assays tests more translatable and economical due to their high throughput and low cost.
7.1. Introduction

In vivo, cancer cells migrate through complex, confining three-dimensional (3D) microenvironments, including tunnel-like regions in the interstitial spaces of the tumor stroma (Alexander et al., 2008; Gritsenko et al., 2012; Weigelin et al., 2012; Stroka et al., 2014c), circulatory or lymph vessels (Alexander et al., 2013), or vasculature of target organs (Alexander et al., 2013; Naumov et al., 1999). These migration tracks are topographically complex, with pores ranging in size from <1 μm to 20 μm in diameter (~10-1000 μm² pore area) (Wolf et al., 2009), and they are often bordered by thick, aligned collagen bundles that create “paths of least resistance” for cell migration (Alexander et al., 2008). Mounting evidence suggests that cells adopt distinct signaling pathways to optimize cell locomotion in different physical microenvironments (Friedl et al., 2010; Sanz-Moreno et al., 2008; Yamazaki et al., 2009; Sanz-Moreno et al., 2010), such as confined versus unconfined spaces (Balzer et al., 2012; Hung et al., 2013; Stroka et al., 2014b; Liu et al., 2015).

In vitro models of cellular-scale migration spaces include printed extracellular matrix (ECM) proteins (Maiuri et al., 2012; Doyle et al., 2009) and microchannel devices (Irimia et al., 2009; Rolli et al., 2010; Tong et al., 2012; Stroka et al., 2014a).
both models, an elongated, polarized cell morphology accompanies persistent cell migration. One possible driver of migration along tracks in the tumor microenvironment, in target organs, and in these bioengineering models is contact guidance. Contact guidance describes the phenomenon in which cells align to topographic features of a substrate (Londono et al., 2014; Saito et al., 2014) and has typically been studied on grooved substrates with pitch less than the width of a cell, which is significantly smaller than cell-scale topographical cues found in vivo. Here, we examined contact guidance in MDA-MB-231 breast adenocarcinoma cells migrating within engineered microchannels imposing various degrees of confinement on the cells (e.g., cell contact with two versus four walls of a microchannel). Migration within microchannels was directly compared to migration on laterally confining 2D printed protein lines and unconfined 2D surfaces. The microcontact printed lines used here were wider than those used in Chapters 5 and 6 (20 µm vs. 6 µm). We found that, for these cells, confinement within microchannels drives directional cell migration, with an increase in persistence and a reduction in cellular protrusivity. Interestingly, lateral confinement on microcontact printed surfaces and three-dimensional confinement within microchannels led to divergent morphological characteristics.

7.2. Materials and Methods

7.2.1. Cell lines and cell culture. MDA-MB-231 human breast carcinoma cells were grown to 70-90% confluency in Dulbecco’s Modified Eagle Medium (DMEM) with 4.5 g/l glucose, L-glutamine, and sodium pyruvate (Corning cellgro, Manassas, VA, USA) and supplemented with 10% (v/v) fetal bovine serum (FBS; Life Technologies Gibco, Carlsbad, CA, USA) and 1% (v/v) penicillin/streptomycin (100 units penicillin, 100
µg/ml streptomycin; Gibco). Cells were maintained in a humidified incubator at 37°C, 5% CO₂. Cells were subcultured every 3-5 days following detachment from cell culture dishes by the addition of 0.05% trypsin-EDTA (Gibco) and passed at ratios of 1:2-1:6.

7.2.2. Fabrication of a microfluidic device for examination of cell migration in multiple topographic regimes. To define the topography of the microenvironment in which cells migrated, an array of microchannels was fabricated between mutually perpendicular feeder channels using multilayer photolithography. The straight feeder regions of the microchannels were 3 µm or 20 µm wide and 10 µm tall; feeder channels bifurcated to branch channels, but cells were only tracked in the straight region of the channels in this study (see below). Designs were produced in AutoCAD (Autodesk, McLean, VA, USA) and transferred to chrome-on-glass darkfield photolithography masks (Photoplot Store, Colorado Springs, CO, USA). A scale schematic of the migration device is shown in Figure 7-1.

Molds for the microfluidic devices were fabricated using multilayer photolithography. SU-8 3010 negative photoresist (Microchem, Newton, MA, USA) was spun to a thickness of 10 µm on a mechanical grade silicon wafer (University Wafer, South Boston, MA, USA), soft baked, and exposed through a mask defining the Y-shaped microchannels on an EVG620 mask aligner (EVG, Austria). Following a post-exposure bake, the photoresist was developed with SU-8 developer, and the patterned wafer was rinsed with isopropanol. A layer of SU-8 3025 was spun on top of the microchannels to a thickness of 50 µm, soft baked, and exposed through a mask defining the inlet and outlet channels of the device. The new layer of photoresist was post-
exposure baked and developed. The completed wafer was hard baked for 10 min at 150°C and treated with (tridecafluoro-1,1,2,2,-tetrahydrooctyl)-1-trichlorosilane (Pfaltz & Bauer, Waterbury, CT, USA) overnight.

The final microfluidic devices were formed using replica molding with polydimethylsiloxane (PDMS, Sylgard 184 kit, Dow Corning, Midland, MI, USA). PDMS prepolymer and crosslinker were mixed at a 10:1 ratio, poured over the wafer, degassed, and cured at 85°C for 2 h. PDMS devices were peeled from the wafer mold and diced. Identical PDMS devices were used to create 2D or 3D Y-channel topographies for microfluidic template printing or confined migration assays, respectively, as described below.
Figure 7-1. Design of microfluidic device and microcontact printed surfaces. To-scale schematic of the microfluidic device used for migration studies and as a template to print collagen type I on glass coverslips. The device was fabricated via replica molding with PDMS and was attached to a glass slide or coverslip prior to cell seeding. Insets show specific regions of the device. Unconfined 2D surfaces were available in the cell seeding region below the microchannel entrances. Microchannels were arrayed between larger cell seeding and medium channels and are shown in the lower inset. Microchannels consisted of 3 µm-wide or 20 µm-wide feeder channels bifurcating to 3 µm-wide or 20 µm-wide branch channels. All microchannels were 10 µm in height. Alternatively, collagen type I was printed in the same projected geometry as that of the microchannels. Deposited collagen is shown in the epifluorescence image. In experiments discussed in Chapter 7, cells were only tracked in the straight feeder regions of the microchannels or printed regions and did not interact with the bifurcation. Scale bars in all phase and epifluorescent images are 50 µm.
7.2.3. Microchannel cell migration experiments. PDMS devices and glass coverslips or slides (75 mm x 25 mm) were cleaned with ethanol and DI water and exposed to oxygen plasma in a Harrick PDC-32G plasma cleaner (Harrick Plasma, Ithaca, NY, USA) at 18 W for 2 min following a 5 min chamber evacuation. PDMS devices were irreversibly bound to the glass slide or coverslip and immediately filled with a solution of 20 µg/ml collagen type I (BD Biosciences, San Jose, CA, USA). Devices were coated by absorption for 1 h at 37°C. The device was washed with DPBS following the removal of the coating solution. Cells were harvested from tissue culture dishes by the addition of 0.05% trypsin-EDTA. Following quenching by an initial resuspension in growth medium, cells were prepared for seeding by two sequential washes in serum-free medium (DMEM with 1% (v/v) P/S, no FBS).

MDA-MB-231 cells were then resuspended to 1-2x10⁶ cells/ml and seeded at the base of the channels by addition of 50 µl of the cell solution to the cell inlet well (50,000-100,000 total cells) of the device, with 20 µl of serum-free media on the opposite side of the microchannels to prevent convective flow of cells into the channels. Cells adhered at the microchannel entrances for 5-10 min prior to washing of the device with DPBS. The four inlet wells of the device were then filled with 100 µl/well of assay medium.

During migration experiments, cells migrated in the presence of serum (10% FBS), with 100 µl of growth medium pipetted in all inlet wells of the device at the beginning of an experiment. Migration in both the microchannels and on the 2D portions of the device was imaged for up to 24 h on an inverted Nikon Eclipse Ti microscope (Nikon, Tokyo, Japan) with automated controls (NIS-Elements, Nikon) and a 10x/0.45 NA Ph1 objective using time-lapse microscopy. Cells were maintained on a temperature-
and CO_2-controlled stage top incubator (Okolab, Italy or Tokai Hit, Japan) throughout the experiments. In select experiments, cells migrating inside 20 μm-wide feeder microchannels were fixed, stained for F-actin using Alexa Fluor 488 phalloidin, and imaged via confocal microscopy, as described previously (Stroka et al., 2014b).

7.2.4. Microfluidic template printing experiments. PDMS devices and glass coverslips were cleaned with ethanol and DI water. The device was reversibly bound to the coverslip by application of gentle pressure to seal the microchannel outlines to the glass surface. The device was primed with a 5% Alconox solution, washed 3X with DPBS, and filled with 20 μg/ml collagen type I containing 1:500 collagen type I antibody (Sigma C2456, clone COL-1) and 1:1000 Alexa488 (Life Technologies) to mark the printed collagen regions. The collagen solution was absorbed on the coverslip for 1 h at 37°C. The collagen solution was then washed out of the device via a triplicate wash with DPBS, the device was peeled from the coverslip, and the coverslip was washed with DPBS. The slide was immersed in 2% (w/v) Pluronic F-127 (Sigma-Aldrich P2443) solution for 1 h at room temperature to backfill nonprinted regions and make them nonadhesive to cells. The slide was washed with DPBS 3X and stored for up to 48 h at 4°C prior to cell seeding. To seed cells, MDA-MB-231 cells were prepared identically as for microchannel experiments and resuspended to 400,000-800,000 cells/ml. A volume of 100 μl (40,000-80,000 total cells) was then pipetted over the printed regions. Medium reservoirs surrounding the printed regions were filled with growth medium, and cells were allowed to settle and adhere to the protein patterns for 1-2 h. The solution was then aspirated, and the surface was washed with DPBS to remove non-adherent cells. The
medium reservoir was filled with medium, and cells were imaged every 10 min for up to 26 h using time-lapse microscopy as described above.

7.2.5. Cell tracking. Cell paths were manually tracked every 10 min in ImageJ (NIH, Bethesda, MD, USA) using the MTrackJ plugin (Meijering et al., 2012). Additionally, cells were traced every 30 min using the polygon ROI function in ImageJ, and shape factors were measured. All cells that fully entered the microchannels or microcontact printed regions for at least 1 h were tracked for the duration of their migration in the straight region of the microchannel or printed surface, beginning when the cell was fully within the microchannel or on the printed design. For microcontact printing experiments, only cells that seeded on or entered the straight region of the microcontact printed lines were considered. If the cell exited the channel or entered the bifurcation region, tracking was discontinued. Cells were tracked while on the straight region of the channels. Dividing cells were not tracked.

All cell path and shape calculations were made using a custom-written Matlab program with cell position and shape data as the input. Averages of measurements in speed or shape factors were calculated by taking the mean of those measurements for one cell. The coefficients of variation in those measurements for a given cell were also calculated. Persistence was calculated by dividing the net displacement of the cell by the total distance traveled by the cell over the course of tracking. Shape factors were calculated similarly using the ImageJ Measure ROI function. Therefore, each point on the plots provided represents the average value of that metric for a given cell, or the variance in that metric measured over the course of tracking for that cell. Individual measurements
were grouped across biological repeats to obtain characteristic distributions of cell speed, persistence, and shape factors for each microenvironment and treatment described.

7.2.6. **Statistics.** Cells from experiments for a given set of conditions (e.g., channel geometry, 2D environment, etc.) were grouped prior to analysis. A minimum of 3 independent experiments were run for each condition. For all statistical analysis, data were analyzed for normality using the D’Agostino-Pearson omnibus normality test. Samples with Gaussian distributions were compared via unpaired t-test or one-way ANOVA with post-hoc Tukey’s multiple comparisons test. Samples with non-Gaussian distributions were compared using an unpaired Mann-Whitney test or Kruskal-Wallis test with Dunn’s multiple comparisons test. Calculations were performed using GraphPad Prism 6.

7.3. **Results**

7.3.1. **Confinement promotes persistent migration of MDA-MB-231 cells.** To examine how the microenvironment impacts cell migration, a variety of substrates with cellular-scale feature sizes were engineered. MDA-MB-231 cells migrated within collagen type I-coated PDMS microchannels with straight “feeder” channels of 3 µm or 20 µm in width and 10 µm in height (Fig. 7-1). Alternatively, a PDMS mold of the microchannels was used as a template to print collagen type I on a glass slide in the same projected geometry as the microchannels (Fig. 7-1). The areas surrounding the patterned features were made inaccessible to cell adhesion by treatment with Pluronic F-127, an amphiphilic block copolymer that forms a stable anti-adhesive coating on cell culture substrates, repelling cell attachment for ~1 week (Raman et al., 2013). Confined
migration was compared to unconfined, 2D migration in a large, open section of the microfluidic device (Fig. 7-1).

We observed that different forms of confinement produced qualitatively and quantitatively distinct migration patterns for the MDA-MB-231 cell line (Fig. 7-2). Cells on unconfined 2D surfaces migrated following the typical mesenchymal protrusion-retraction cycle (Ananthakrishnan et al., 2007) with frequent direction changes (Fig. 7-2A). On the other hand, cells in 3 µm-wide microchannels were shaped by the microchannel geometry and assumed a pill-like morphology (Fig. 7-2A). When partially confined, cells displayed distinct substratum-dependent behaviors. In 20 µm-wide microchannels, cells preferentially moved along one side wall, typically elongating and migrating with a single leading protrusion (Fig. 7-2, Fig. 7-3). These cells spread equally on the floor and the side wall of the channel, spanning approximately 10 µm in each wall (Fig. 7-3). In contrast, cells on printed protein lines of identical dimensions and ligand coating as the microchannels spread to fill the adhesive region and migrated with a broad, dominant leading lamellipodium (Fig. 7-2A).
Figure 7-2. The physical microenvironment affects the persistence of MDA-MB-231 cell migration through changes in cell shape and morphodynamics. (A) Phase contrast images of MDA-MB-231 cells migrating on 2D unconfined surfaces, or inside Y-shaped microchannels with either 3 µm- or 20 µm-wide feeder channels bifurcating to 3 µm- and 20 µm-wide branches, or on Y-shaped printed lines with a feeder region of 20 µm in width bifurcating to 3 µm- and 20 µm-wide branches. All surfaces were coated with collagen type I (20 µg/ml). Arrowheads illustrate the positions of given cells at prescribed time points. Scale bar represents 50 µm. (B) Average persistence ratios, (C) average speeds, and (D) number of direction changes in the y-
direction per distance travelled by the cell. Parameters were quantified in the straight feeder regions of the microchannels or printed lines (prior to the bifurcation). Columns represent population means. Error bars show standard error of the mean. Data points represent values of each metric for one cell. Significance was assessed by Kruskal-Wallis test with post-hoc Dunn’s multiple comparisons test. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001.
Figure 7-3. Confocal reconstruction of MDA-MB-231 cell F-actin architecture in 20 µm-wide feeder microchannels. (A-C) Representative brightfield and confocal images of MDA-MB-231 cells migrating in 20 µm-wide, 10 µm-tall feeder microchannels. Cells were stained for F-actin using Alexa Fluor 488 phalloidin. Confocal images taken at the basal surface of the cell are shown in the x,y plane. Cells were imaged at 0.8 µm axial intervals to generate orthogonal reconstructions in the x,z and y,z planes. The locations of the orthogonal reconstructions are indicated by the white lines in the x,y planes. In panel (B), a 3D surface reconstruction of the indicated cell is shown in the inset. Scale bar represents 20 µm in all panels.
The effect of the local microenvironment on migration was first quantified by calculating cell persistence, defined as the net displacement of a cell divided by the total distance traveled by the cell over the course of tracking. Persistence is indicative of cell polarization, with highly polarized cells migrating with high persistence ratios. Measurements were made in the straight feeder region of each setup. The microenvironment significantly modulated cell persistence (Fig. 7-2B) while modestly impacting cell speed (Fig. 7-2C). Partial or full confinement within microchannels increased migration persistence when compared to MDA-MB-231 cells on unconfined 2D surfaces. No significant difference in persistence was noted between unconfined 2D surfaces and printed lines at this line width (20 μm). Persistence was significantly higher for fully confined versus partially confined cells, indicating that increasing confinement enhances cell persistence, which is in line with prior work (Pathak et al., 2012). Furthermore, front-to-back directional changes were reduced in microchannels compared to those on flat surfaces, with the fewest direction changes occurring in full confinement (Fig. 7-2D).

7.3.2. Changes in MDA-MB-231 cell morphodynamics underlie confinement-induced cell persistence. To gain insights into how the microenvironment modulated cell persistence, we traced the projected outline of migrating MDA-MB-231 cells over 30-min intervals (Fig. 7-4). Measurements of cell outlines allow for calculations of shape factor that provide quantitative data that can be used to define different modes of cell migration. Representative tracks and shape factors are shown in Figure 7-4. As polarized cells are characterized by dominant protrusions and seem to repress nascent protrusions
to maintain migration in one direction (Pankov et al., 2005; Petrie et al., 2009a; Riching et al., 2014), we hypothesized that more persistent cells would have fewer protrusions and decreased morphodynamics compared to less persistent cells. Global cell protrusivity was measured by calculating the solidity of the cell outline. Solidity is the ratio of the area to the convex area of the cell shape (Pasqualato et al., 2013). Lower solidity corresponds to cells with more protrusions (Fig. 7-4J). Confined cells, which typically moved with only one dominant protrusion, had significantly higher solidity than cells on unconfined 2D surfaces (Fig. 7-5A). Variances in solidity measurements were also higher in unconfined 2D migration than in confinement, indicating increased morphodynamics in 2D as cells more fully explored their environment and made directional changes (Fig. 7-5B). Collectively, MDA-MB-231 cells in confinement both moved more persistently and with fewer protrusions and decreased morphodynamics compared to unconfined cells.

We further hypothesized that the high degrees of persistence noted in confined MdA-MB-231 cells would manifest in nearly constant angular orientation of a migrating cell. While average fit elliptical angles were identical (Fig. 7-5C), the coefficient of variation of the angle of an ellipse fit to a cell over the course of migration was markedly higher in unconfined 2D than in confinement (Fig. 7-5D; also see Fig. 7-4H). In confinement, this reduced variance in orientation was presumably due to persistent alignment along topographical features and a reduced exploration of the microenvironment. Measurements of other shape factors are shown in Figure 7-6 and further support the conclusion that increased persistence is accompanied by a reduction in cell exploration of the microenvironment. Specifically, confinement caused MDA-MB-
231 cell elongation (increases in aspect ratio in confinement; Fig. 7-6A) and a reduction in cell width (decreases in projected area and minor axis length; Fig. 7-6D,J). These differences were most pronounced in 3 µm-wide microchannels, where cells were in contact with all four microchannel walls. Interestingly, variations in cell length were also minimized in 3 µm-wide microchannels. MDA-MB-231 cells moved with almost constant length in these channels instead of protruding and retracting (compare coefficient of variation in aspect ratio and major axis length among microenvironments; Fig. 7-6B,I).
Figure 7-4. Shape factors of MDA-MB-231 cells in different physical microenvironments.

Representative time lapse images of MDA-MB-231 cells migrating (A) on a 2D surface, (B) inside 3 µm-wide microchannels, (C) inside 20 µm-wide microchannels, and (D) on 20 µm-wide printed collagen type I lines. Microchannels were 10 µm in height. All surfaces were coated with...
collagen type I (20 µg/ml). Scale bars represent 50 µm. Measurements of (E) speed, (F) projected area, (G) cell minor axis length, (H) fit elliptical angle, (I) aspect ratio, (J) solidity, and (K) circularity for the cells shown in (A)-(D) at prescribed time points. Average values and variances of each metric across the time of tracking are noted to the side of each plot.
Figure 7-5. Physical confinement reduces the extent and dynamics of protrusions and causes persistent alignment for MDA-MB-231. Shape factors of migrating MDA-MB-231 cells were quantified in the feeder channel for each physical microenvironment. Average (A) solidity, (B) coefficient of variation in solidity, (C) fit elliptical angle, and (D) coefficient of variation in fit elliptical angle of MDA-MB-231 cells migrating in all microenvironments tested. Columns represent population means. Error bars show standard error of the mean. Data points represent values of each metric for one cell. Significance was assessed by Kruskal-Wallis test with post-hoc Dunn’s multiple comparisons test. **, p<0.01; ***, p<0.001; ****, p<0.0001.
Figure 7-6. The shape and morphodynamics of migrating MDA-MB-231 cells are modulated by the microenvironment. Shape factors of migrating MDA-MB-231 cells were quantified in the feeder channel for each physical microenvironment. Average (A) aspect ratio, (B) coefficient of variation in aspect ratio, (C) coefficient of variation in speed, (D) projected area, (E) coefficient of variation in area, (F) circularity, (G) coefficient of variation in circularity, (H) cell major axis
length, (I) coefficient of variation in major axis length, (J) cell minor axis length, and (K) coefficient of variation in minor axis length of MDA-MB-231 cells migrating in all microenvironments tested. Columns represent population means. Error bars show standard error of the mean. Data points represent values of each metric for one cell. Significance was assessed by Kruskal-Wallis test with post-hoc Dunn’s multiple comparisons test. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001.
7.4. Discussion

Understanding how cells respond to different physical microenvironments is crucial for developing strategies that control, manipulate, promote, or stop cell motility in vivo. Mounting evidence suggests that the local topography of the tumor microenvironment regulates the modes of cell migration (Nguyen-Ngoc et al., 2012; Kedrin et al., 2008). Cell invasion from primary tumors is associated with bundled ECM protein fibers aligned radially to the tumor interface (Provenzano et al., 2006). Here, we studied how guidance cues provided by different engineered structures potentiated the persistent migration of MDA-MB-231 cells.

Specifically, we employed a comprehensive quantitative analysis to directly compare MDA-MB-231 cell migration in different microenvironments: unconfined 2D substrates, laterally confined 2D surfaces, and confined 3D microchannels of the length scales typically found in vivo (Weigelin et al., 2012; Wolf et al., 2009). Confinement drove persistent migration of these cells and was accompanied by a reduction in the abundance and dynamics of protrusions along with a concomitant increase in cell alignment along micron-scale features. In line with our observations, collagen fiber alignment in gels does not affect the migration speed of MDA-MB-231 cells but instead increases persistence through a reduction in protrusions (Riching et al., 2014). The most extensive increase in confinement and decrease in protrusivity were found in highly confining 3 µm-wide, 10 µm-tall microchannels. Intermediate morphology was observed in 20 µm-wide microchannels, and cell morphology was often indistinguishable from that in 2D when cells migrated on 20 µm-wide printed lines. These results are in line with our
findings in Chapter 6, which demonstrated increasing cell persistence as confinement increased.

Confinement likely potentiates persistent cell migration through contact guidance, with cells aligning along and following topographic features of their microenvironment. Indeed, we observed persistent alignment of MDA-MB-231 cells along the various types of confining substrates tested. The functional impact of contact guidance on cell decision making is explored in the next chapter.
Chapter 8

THE INTERPLAY OF THE PHYSICAL MICROENVIRONMENT, CONTACT GUIDANCE, AND CELL SIGNALING IN CELL DECISION MAKING

8.1. Introduction

While straight, tunnel-like microchannels provide a first-order approximation of the space tracks found \textit{in vivo}, they do not account for the plasticity of the tumor microenvironment itself, which further promotes cell invasion through tracks that dynamically appear and change as the disease progresses (Friedl et al., 2011). Therefore, models of the tumor microenvironment should include both confinement and anisotropic physical features to reflect the variability of tissues in the body. Delineating microenvironment-dependent migration mechanisms underlying topology-driven cell migration and decision making at tissue/vascular junctures is important for understanding cell trafficking during cancer metastasis and enabling engineering changes to the microenvironment to abrogate metastatic spread.

While much is known about how tumor cells migrate, little is known about how they make decisions to navigate complex microenvironments. A panel of epithelial cells migrating through confined microchannel “mazes” locally consume growth factors to create autologous gradients that assist in finding the shortest migration path (Scherber et al., 2012). Similarly, leukocytes confined in microchannels encountering bifurcations
preferentially enter the channel of least hydraulic resistance (Prentice-Mott et al., 2013). Along these lines, MDA-MB-231 breast cancer cells migrating inside confined microchannels preferentially choose the wider branches, even when actomyosin contractility is inhibited (Mak et al., 2014). However, all these studies were performed using only a single feeder channel width, which was smaller than the cell diameter. As such, these studies do not account for the range of pore sizes found in the body (Wolf et al., 2009). It is conceivable that the decision making processes may depend on the size of the feeder channel. In addition, the molecular constituents mediating cell decision making and response to topographical cues (that is, contact guidance) of various sizes are not known.

Here, we examined how contact guidance mediated decision making in MDA-MB-231 breast adenocarcinoma and HT1080 fibrosarcoma cells migrating within engineered microchannels and microcontact printed surfaces. When polarized cells were challenged to make a decision between unequally-sized microchannels at a bifurcation point, the degree and type of confinement were key inputs on decision making. For MDA-MB-231 cells, decision-making from 3D microchannels depended on the length scales of the feeder-channel. MDA-MB-231 cells confined in narrow feeder-channels preferentially entered wider branches at bifurcations, as did cells laterally confined on microcontact printed lines. In contrast, in feeder-channels wider than the cell body, cells elongated along one side-wall and were contact guided to the contiguous branch channel independently of its width. For both cell lines tested, inhibition of cellular contractility or concurrent knockdown of non-muscle myosin isoforms IIA and IIB decreased contact
guidance. Conversely, inhibition or knockdown of Cdc42 promoted contact guidance-mediated decision-making.

8.2. Materials and Methods

8.2.1. Cell lines and siRNA experiments. MDA-MB-231 human breast carcinoma and HT1080 human fibrosarcoma cells were grown to 70-90% confluency in Dulbecco’s Modified Eagle Medium (DMEM) with 4.5 g/l glucose, L-glutamine, and sodium pyruvate (Corning cellgro, Manassas, VA, USA) and supplemented with 10% (v/v) fetal bovine serum (FBS; Life Technologies Gibco, Carlsbad, CA, USA) and 1% (v/v) penicillin/streptomycin (100 units penicillin, 100 µg/ml streptomycin; Gibco). Cells were maintained in a humidified incubator at 37°C, 5% CO₂. Cells were subcultured every 3-5 days following detachment from cell culture dishes by the addition of 0.05% typsin-EDTA (Gibco) and passed at ratios of 1:2-1:6. During select experiments, cells seeded in the devices were incubated with the following pharmacological agents or their appropriate vehicle control (VC): 50 µM (-)-blebbistatin (Sigma-Aldrich, St. Louis, MO, USA; VC is DMSO); 20 µM ML141 (Santa Cruz Biotechnology, Dallas, TX, USA; VC is DMSO); or 1, 10, or 50 uM NSC23766 (EMD Millipore, Billerica, MA, USA; VC is water).

In separate experiments, select proteins involved in cell migration and polarity were knocked down via siRNA transfection with Lipofectamine 2000 (Life Technologies). All siRNAs were from Santa Cruz Biotechnology: control-A (sc-37007), MYH9 (sc-61120), MYH10 (sc-61122), RhoA (sc-29471), Rac 1 (sc-36351), Cdc42 (sc-29256), and β1 integrin (sc-35674). For knockdown, a total of 200,000 cells were seeded
in the well of a 6-well plate and spread overnight in growth medium. Targeting or control siRNA at a concentration of 10 µM in manufacturer-provided dilution buffer was diluted with Lipofectamine 2000 in Opti-MEM transfection medium (Life Technologies) at a ratio of 9.33 µl Lipofectamine:13.33-26.67 µl siRNA:400 µl Opti-MEM. 200 µl of the transfection cocktail (60-120 pmol siRNA/well) was added to plated cells immersed in 800 µl of Opti-MEM. The resulting transfection medium contained 0.06-0.12 pmol siRNA/ml transfection medium. After 6 h in the incubator, 1 ml/well of DMEM with 20% FBS, 2% P/S was added to each well. Cells were washed with DPBS and media was replaced with regular growth medium 24 h after initial siRNA addition. Cells were used in migration experiments or examined via Western blot 48 hours after transfection.

8.2.2. Western blot analysis. Western blots analysis was performed as described previously (Chen et al., 2013b; Chen et al., 2012; Wang et al., 2014). Whole cell lysate from MDA-MB-231 cells treated with control-A siRNA or target-specific siRNA were separated using either a 4-12% Bis-tris or a 3-8% Tris-acetate SDS-PAGE gel (Bio-Rad, Hercules, CA, USA) under reducing conditions. Proteins were transferred to an Immuno-blot nitrocellulose membrane (Sigma-Aldrich) and blocked for 20 min using StartingBlock blocking buffer (Life Technologies). Membranes were stained with either anti-myosin IIA (M8064, Sigma-Aldrich), anti-myosin IIB (clone N-17, Santa Cruz), anti-RhoA (clone 26C4, Santa Cruz), anti-Rac 1 (clone 23A8, EMD Millipore), anti-β1 (clone N-20, Santa Cruz), or anti-Cdc42 (clone P1, Santa Cruz) antibody. An anti-actin (Ab-5, BD) antibody was used as a loading control. Membranes were rinsed with TBS/0.1% Tween 20 and incubated with the appropriate horseradish peroxidase (HRP)-
conjugated secondary antibody. SuperSignal West Pico chemiluminescent substrate (Life Technologies) was used to develop the HRP-conjugated antibody-stained immunoblots.

8.2.3. Fabrication of a microfluidic device for examination of cell migration in multiple topographic regimes. To define the topography of the microenvironment in which cells migrated, an array of microchannels was fabricated between mutually perpendicular feeder channels using multilayer photolithography. The microchannels were “Y”-shaped, with 3 μm-wide or 20 μm-wide by 10 μm-tall feeder channels bifurcating to 20 μm-wide or 3 μm-wide by 10 μm-tall branch channels. End-to-end channel length was set at either 190 μm or 390 μm. Designs were produced in AutoCAD (Autodesk, McLean, VA, USA) and transferred to chrome-on-glass darkfield photolithography masks (Photoplot Store, Colorado Springs, CO, USA). A scale schematic of the migration device is shown in Figure 8-1. This was the same device used as in Chapter 7, but cells were followed through the bifurcation point in this study.

Molds for the microfluidic devices were fabricated using multilayer photolithography. SU-8 3010 negative photoresist (Microchem, Newton, MA, USA) was spun to a thickness of 10 μm on a mechanical grade silicon wafer (University Wafer, South Boston, MA, USA), soft baked, and exposed through a mask defining the Y-shaped microchannels on an EVG620 mask aligner (EVG, Austria). Following a post-exposure bake, the photoresist was developed with SU-8 developer, and the patterned wafer was rinsed with isopropanol. A layer of SU-8 3025 was spun on top of the microchannels to a thickness of 50 μm, soft baked, and exposed through a mask defining the inlet and outlet channels of the device. The new layer of photoresist was post-
exposure baked and developed. The completed wafer was hard baked for 10 min at
150°C and treated with (tridecafluoro-1,1,2,2,-tetrahydrooctyl)-1-trichlorosilane (Pfaltz &
Bauer, Waterbury, CT, USA) overnight.

The final microfluidic devices were formed using replica molding with
polydimethylsiloxane (PDMS, Sylgard 184 kit, Dow Corning, Midland, MI, USA).
PDMS prepolymer and crosslinker were mixed at a 10:1 ratio, poured over the wafer,
degassed, and cured at 85°C for 2 h. PDMS devices were peeled from the wafer mold and
diced. Identical PDMS devices were used to create 2D or 3D Y-channel topographies for
microfluidic template printing or confined migration assays, respectively, as described
below.
Figure 8-1. Design of microfluidic device and microcontact printed surfaces for cell decision making studies. (A) To-scale schematic of the microfluidic device used for migration studies and as a template to print collagen type I on glass coverslips. The device was fabricated via replica molding with PDMS and was attached to a glass slide or coverslip prior to cell seeding. Insets show specific regions of the device. Unconfined 2D surfaces were available in the cell seeding region below the microchannel entrances. Microchannels were arrayed between larger cell
seeding and medium channels and are shown in the lower inset. Microchannels consisted of 3 
µm-wide or 20 µm-wide feeder channels bifurcating to 3 µm-wide or 20 µm-wide branch 
channels. All microchannels were 10 µm in height. Alternatively, collagen type I was printed in 
the same projected geometry as that of the microchannels. Deposited collagen is shown in the 
epifluorescence image. Scale bars in all phase and epifluorescent images are 50 µm. (B) In select 
experiments, shorter microchannels in which the bifurcation occurred 90 µm (as opposed to 265 
µm) from the microchannel entrance were used. Scale bar represents 50 µm.
8.2.4. Microchannel and microfluidic template printing cell migration experiments.

Microchannel and microfluidic template printing experiments were performed as described in Chapter 7. For experiments in which a pharmacological agent was used, medium containing the agent or appropriate vehicle control was added to the microfluidic device following cell seeding and prior to imaging.

8.2.5. Cell tracking. Cell paths were manually tracked every 10 min in ImageJ (NIH, Bethesda, MD, USA) using the MTrackJ plugin (Meijering et al., 2012). All cells that fully entered the microchannels or microcontact printed regions for at least 1 h were tracked for the duration of their migration, beginning when the cell was fully within the microchannel or on the printed design. For microcontact printing experiments, only cells that seeded on or entered the base region of the microcontact printed lines were considered. If the cell exited the channel, tracking was discontinued. If cells reached the branch region (see Fig. 8-1), tracking was discontinued at the maximum distance away from the entrance (that is, cells that reached the branch region were not tracked if they turned around). Dividing cells were not tracked, and tracks for cells that were blocked at the bifurcation by the presence of an occluding cell were disregarded. Position measurements were made with respect to the midpoint of the channel entrance (see Fig. 8-1). In select experiments, cell shape was also tracked (see Chapter 7).

All cell path calculations were made using a custom-written Matlab program with cell position as the input. Averages of measurements in speed were calculated by taking the mean of those measurements for one cell. The coefficients of variation in those measurements for a given cell were also calculated. Persistence was calculated by
dividing the net displacement of the cell by the total distance traveled by the cell over the course of tracking. Therefore, each point on the plots provided represents the average value of that metric for a given cell, or the variance in that metric measured over the course of tracking for that cell. Individual measurements were grouped across biological repeats to obtain characteristic distributions of cell speed and persistence for each microenvironment and treatment described.

8.2.6. Statistics. Cells from experiments for a given set of conditions (e.g., channel geometry, inhibitor, knockdown) were grouped prior to analysis. A minimum of 3 independent experiments were run for each condition. All experiments using an inhibitor or knockdown were run side-by-side with cells treated with the inhibitor vehicle or transfected with non-targeting control siRNA, respectively. For all statistical analysis, data were analyzed for normality using the D’Agostino-Pearson omnibus normality test. Samples with Gaussian distributions were compared via unpaired t-test or one-way ANOVA with post-hoc Tukey’s multiple comparisons test. Samples with non-Gaussian distributions were compared using an unpaired Man-Whitney test or Kruskal-Wallis test with Dunn’s multiple comparisons test. Calculations were performed using GraphPad Prism 6. Fractions of cells to branch channels of various widths or fractions of cells contact guided were analyzed using the two-population proportion z test. 95% confidence intervals for the proportions measured were calculated using the “exact” method of Clopper and Pearson in GraphPad.
8.3. Results

8.3.1. The distinct roles of confinement and contact guidance in MDA-MB-231 cell decision making at bifurcations. To test how dimensionality and varying degrees of confinement affect cell decision making, we challenged persistently migrating MDA-MB-231 cells with a bifurcation in which the feeder region split to wide (20 µm-wide) or narrow (3 µm-wide) branch regions. Feeder regions consisted of 3 µm-wide or 20 µm-wide, 10 µm-tall microchannels bifurcating to 10 µm-tall branch channels (all coated with 20 µg/ml collagen type I), or of 20 µm-wide printed collagen type I lines bifurcating to branch lines. Representative time lapse images of cells making decisions from each feeder channel environment are shown in Figure 8-2. Interestingly, the decision of which branch to enter was dependent on the microenvironment of the feeder region (Fig. 8-3). Cells migrating from 20 µm-wide printed lines preferentially entered the wider (20 µm in width) branch, as did cells moving from 3 µm-wide feeder microchannels. Specifically, cells preferentially entered the wider branch channel (~75%) (Fig. 8-3) when migrating from 3 µm-wide feeder channels in which the narrow width was set at 3 µm while the wider branch width increased from 3 µm to 6, 10, 15, or 20 µm (Fig. 8-3B,C)
Figure 8-2. Representative images of MDA-MB-231 cells making decisions from various feeder channel microenvironments. Phase contrast images of MDA-MB-231 cells migrating on 2D unconfined surfaces, or inside Y-shaped microchannels with either 3 µm- or 20 µm-wide feeder channels bifurcating to 3 µm- and 20 µm-wide branches, or on Y-shaped printed lines with a feeder region of 20 µm in width bifurcating to 3 µm- and 20 µm-wide branches. All surfaces were coated with collagen type I (20 µg/ml). Arrowheads illustrate the positions of given cells at prescribed time points. Scale bar represents 50 µm.
**Figure 8-3. The distinct roles of confinement and contact guidance in MDA-MB-231 cell decision making at bifurcations.** (A) Fraction of MDA-MB-231 cells from each microenvironment entering the 20 µm-wide branch at bifurcations. (B) Fraction of cells from a 3 µm-wide feeder microchannel migrating into the right branch. The width of the right branch channel varied from 3, 6, 10, 15, or 20 µm. The width of the left hand branch was set at 3 µm. In
(A) and (B), data represent fraction of cells. Error bars show the 95% confidence intervals of these fractions. Significance was assessed by the two-population proportion z-test with respect to the 20 µm-wide feeder channel (panel A) or the 3 µm-wide right branch channel (panel b). †, p<0.1; *, p<0.05; **, p<0.01; ***, p<0.001. (C) Phase contrast images of 3 µm-wide feeder microchannels bifurcating to branch microchannels of various widths. Microchannels were on a single microfluidic device and were 10 µm in height. Scale bar represents 50 µm. (D) Ratio of cell minor axis length to feeder region width. Measurements were made in feeder region for all cells making a decision at the bifurcation. ****, p<0.0001 by unpaired t-test with Welch’s correction. (E) Schematic of cell paths for a contact guided and a non-contact guided cell. Cells were scored as contact guided if they did not cross the midline of the channel while in the bifurcation region of the device. The bifurcation region (box in image) is defined as the area within one cell length away from the bifurcation. Scale bar represents 50 µm. (F) Fraction of contact guided cells in microchannels versus printed lines. Data represent overall fraction, and bars show 95% confidence intervals of these fractions. Comparisons between fractions of contact guided cells in microchannels and on printed lines were assessed using the two-population proportion z test. *, p<0.05.
In marked contrast, MDA-MB-231 cells migrating from 20 µm-wide feeder microchannels displayed no bias in branch decision, equally entering both the narrow (3 µm) and wide (20 µm) branch channels (Fig. 8-3A). Although NIH3T3 cell preference for larger microcontact-printed areas has been demonstrated previously (Chang et al., 2013), to our knowledge, this is the first demonstration of pore size-independent cell decisions. We noted that the vast majority (>80%) of MDA-MB-231 cells spread and moved preferentially along one side wall of the 20 µm-wide feeder channels before entering the contiguous branch channel on that side, indicating cell contact guidance along a topographical feature on one side of the cell (Fig. 8-2, middle panels). This occurred even when one branch of the microchannel terminated in a dead end instead of an outlet from the microchannel (Fig. 8-4). We thus posited that, at least for this cell line, contact guidance is responsible for decision making from 20 µm-wide feeder microchannels, where cell elongation along a single side wall repressed cell lamellipodia and restricted exploration of the microenvironment at the bifurcation. This is substantiated by the lower minor axis length (10.5±0.4 µm versus 16.8±1.0 µm) and spread area (418±20 µm² versus 604±47µm²) of MDA-MB-231 cells in microchannels compared to on printed lines.
Figure 8-4. Contact guidance regulates decision making of MDA-MB-231 cells at bifurcations stemming from wide feeder channels. Phase contrast images of MDA-MB-231 cells migrating in the absence of an external chemotactic field inside collagen type I-coated Y-shaped PDMS microchannels. Feeder and branch microchannels of (A) 20 µm and (B) 50 µm in width. All channels were 10 µm tall. The left branch channel had an outlet to the medium channel, while the right branch ended in a dead end. Arrowheads illustrate the positions of given cells at prescribed time points. Scale bar represents 50 µm. (C) Fraction of contact guided MDA-MB-231 and HT1080 cells during the decision making process from 20 µm-wide feeder channels. Data show overall fraction of contact guided cells, and bars show 95% confidence intervals of the fractions. Comparison between cell lines was made using the two-population proportion z test. n.s., difference not statistically significant.
For MDA-MB-231 cells, migration along a single side wall was highly predictive of the decision making from wide feeder microchannels. We analyzed the likelihood of these cells switching from one side wall to the other before choosing a branch within approximately one cell length of the bifurcation (Fig. 8-3E). Cells that did not switch walls were termed “contact guided”. This definition is illustrated schematically in Figure 8-3E. Contact guidance was a powerful predictor of cell destination branch for MDA-MB-231 cells migrating from 20 µm-wide microchannels, in which 80% of cells were contact guided (Fig. 8-3F). To generalize our observations, we examined the migratory behavior of HT1080 fibrosarcoma cells. Indeed, contact guidance was also predictive of decision making for HT1080 cells at a bifurcation (Fig. 8-4C). In contrast, cells on microcontact printed lines typically displayed broad lamellipodia and larger spreading areas (Fig. 8-2); as such, cells were not localized to one edge of the printed area, so their position prior to the bifurcation was not predictive of which branch channel they entered (Fig. 8-3F).

8.3.2. The critical length scales affecting MDA-MB-231 cell decision making at bifurcations from partial versus full confinement. To further explore the topographical determinants of contact guidance-mediated decision making, we studied MDA-MB-231 cell behavior in microchannels in which the bifurcation occurred 90 µm (short channels) instead of 265 µm (long channels) from the feeder entrance (Fig. 8-1B; Fig. 8-5A,B). Interestingly, cells migrating from short feeder channels preferentially entered the wider branch, regardless of feeder channel width (Fig. 8-5C). This is in contrast to the results seen in long feeder channels (Fig. 8-5C).
Figure 8-5. The critical length scales affecting MDA-MB-231 cell decision making at bifurcations from partial versus full confinement. Time lapse images of MDA-MB-231 cells migrating through (A) 20 µm-wide or (B) 3 µm-wide feeder microchannels. The bifurcation occurred 90 µm into the feeder channel. Scale bar represents 50 µm. (C) Fraction of cells entering the 20 µm-wide branch in long (265 µm) versus short (90 µm) channels of prescribed feeder widths. Data represent fraction of cells, and error bars show the 95% confidence intervals of these
fractions. Significance was assessed by the two-population proportion z-test with respect to the long 20 µm-wide feeder channel. *, p<0.05; **, p<0.01. (D) Aspect ratio, (E) circularity, (F) fit elliptical angle, and (G) minor axis length of cells as a function of distance into the feeder channel. Measurements were binned within 20 µm-long regions of the feeder channel. Symbols show average value in each bin, accompanied by standard error of each measurement. Symbols are plotted at the center position of each bin. Measurements made in short (90 µm in length) 20 µm-wide feeder microchannels (dark green) are overlaid on those made in long (265 µm in length) 20 µm-wide microchannels.
We hypothesized that the difference in migration decisions at the bifurcation from short versus long 20 µm-wide feeder channels was a function of incomplete polarization prior to the bifurcation – that is, that cells were not fully elongated and polarized along one side wall of the microchannel by the time the bifurcation occurred. We measured cell shape with respect to position in the microchannels by binning measurements taken within 20 µm-long regions of the feeder channel. Cell aspect ratio increased (Fig. 8-5D) whereas circularity decreased (Fig. 8-5E) with distance into the 20 µm-wide feeder microchannels as cells elongated along a side wall. Tight positioning along a side wall was further evidenced by increased alignment of the fit elliptical angle to the channel wall (Fig. 8-5F) and a progressive decrease in minor axis length (Fig. 8-5G) as cells became more localized along one wall of the feeder channel.

In narrow (3 µm in width) feeder channels, confinement imposed cell shape, so shape factors did not change with distance (Fig. 8-5D-G), and cell decisions were not a function of channel length (Fig. 8-5C). Also, no distance-dependent changes in polarization were observed on printed lines (Fig 8-5D-G). Collectively, these measurements indicate iterative cell polarization as the cell aligned along a microchannel side wall and moved farther into the microchannel under conditions of partial confinement.

8.3.3. The critical role of integrin-mediated adhesion in the contact guidance of MDA-MB-231 and HT1080 cells. In view of the key role of contact guidance in MDA-MB-231 and HT1080 cell decision making, we wished to delineate the mechanisms responsible for cell migration along a topographic feature on one side of the cell.
Integrins are transmembrane proteins involved in binding of cells to extracellular substrates and mechanotransduction (Ross et al., 2013). β1 integrin is responsible for cellular binding to collagen type I (Grzesiak et al., 2011). Knockdown of β1 integrin in MDA-MB-231 cells via siRNA (Fig. 8-6A) drastically decreased the efficiency of cell migration in 20 μm-wide feeder microchannels compared to cells transfected with non-targeting control (Ctrl) siRNA, as evidenced by a lower average migration speed and markedly shorter net cell displacements (Fig. 8-6B-E). Moreover, β1 integrin knockdown significantly decreased the spread area of cells in microchannels (Fig. 8-6F), presumably due to the inability of nascent protrusions to bind and mature. Inefficiencies in cell spreading and elongation were further reflected by a significant decrease in cell aspect ratio upon β1 integrin knockdown (Fig. 8-6G). Importantly, any elongation was not persistent or oriented, as there was a significant increase in the variance of the fit elliptical angle upon knockdown (Fig. 8-6H). Similar to our findings using MDA-MB-231 cells, the speed and persistence of HT1080 fibrosarcoma cells were also significantly reduced in 20 μm-wide feeder microchannels upon knockdown of β1 integrin (Fig. 8-7). Because HT1080 cells migrated much faster than MDA-MB-231 cells, they were able reach the bifurcation, even in the absence of β1 integrin. However, β1-knockdown HT1080 cells were significantly less contact guided than control cells (Fig. 8-7). For these cell lines, β1 integrins are required for efficient contact guidance in feeder microchannels wider than the cell body.
Figure 8-6. The critical role of integrin-mediated adhesion in the contact guidance of MDA-MB-231 cells. MDA-MB-231 cells were transfected with non-targeting control (Ctrl) siRNA or siRNA targeting β1 integrin. (A) β1 integrin knockdown was confirmed via Western blot. (B, C) Time lapse images of cells migrating in 20 µm-wide feeder channels following transfection with (B) Ctrl siRNA or (C) siRNA targeting β1 integrin. Scale bar represents 20 µm. (D) Average speed, (E) displacement, (F) projected area, (G) aspect ratio, and (H) coefficient of variation in fit elliptical angle of cells transfected with Ctrl or β1 integrin siRNA. Parameters were quantified in the straight feeder regions of the microchannels. In (D)-(H), columns represent population means. Error bars show standard error of the mean. Data points represent values of each metric for one cell for n≥28 cells from four independent experiments. *, p<0.05; **, p<0.01; ***, p<0.001 by Mann-Whitney test.
Figure 8-7. The critical role of integrin-mediated adhesion in the contact guidance of HT1080 cells. Wild type (WT) HT1080 cells or HT1080 cells in which β1 integrin was stably knocked down (β1 KD) were assayed in devices with 20 µm-wide feeder channels. (A) Average speed and (B) persistence of WT or β1 KD cells. Parameters were quantified in the straight feeder regions of the microchannels. In (A),(B), columns represent population means. Error bars show standard error of the mean. Data points represent values of each metric for one cell for n≥28 cells from four independent experiments. *, p<0.05; ****, p<0.0001 by unpaired t test (speed) or Mann-Whitney test (persistence). (C) Fraction of contact guided cells during the decision making process. Data show overall fraction of contact guided cells, and bars show 95% confidence intervals of the fractions. Comparisons between fractions of treated and control cells were analyzed using the two-population proportion z test. *, p<0.05.
8.3.4. The differential effects of cell contractility in MDA-MB-231 cell decision making at bifurcations from partial versus full confinement. The involvement of β1 integrins in the contact guidance of MDA-MB-231 cells implicates actomyosin contractility as a player in persistent migration along 20 μm-wide feeder channels. We thus assessed the effects of blebbistatin, a myosin II ATPase inhibitor that interferes with actomyosin contractility (Kovacs et al., 2004), on the contact guidance of MDA-MB-231 cells. Blebbistatin-treated MDA-MB-231 cells were significantly slower (Fig. 8-8A) and less persistent (Fig. 8-8B) than vehicle control (DMSO) cells in 20 μm-wide feeder channels. Importantly, inhibition of cell contractility markedly decreased the fraction of contact guided cells at the bifurcation (Fig. 8-8C). Similar to MDA-MB-231 cells, blebbistatin also significantly decreased the speed, persistence, and fraction of contact guided cells when HT1080 fibrosarcoma cells were used (Fig. 8-9). In contrast, blebbistatin did not affect the speed, persistence, or decision making of MDA-MB-231 cells migrating inside fully confining (3 μm-wide) feeder channels (Fig. 8-8D-F).
Figure 8-8. The differential effects of cell contractility in the contact guidance and decision making of MDA-MB-231 cells at bifurcations from partial versus full confinement. MDA-MB-231 cells were treated with 50 µM blebbistatin or vehicle control. In select experiments, cells were transfected with Ctrl siRNA or siRNA targeting myosin IIA (MYH9) and myosin IIB (MYH10). (A) Average speeds and (B) persistence ratios quantified in the 20 µm-wide feeder microchannels for prescribed pharmacological and molecular interventions. (C) Fraction of contact guided cells during the decision making process from 20 µm-wide feeder channels. (D) Average migration speed and (E) persistence of cells treated with 50 µM blebbistatin or vehicle control in 3 µm-wide feeder channels. (F) Fraction of cells migrating in 3 µm-wide feeder channels that enter 20 µm-wide branch channels in the presence or absence of blebbistatin. (G) Knockdown of myosin isoforms was confirmed via Western blot, with actin as loading control. Panel shows two blots from the same cell lysate immunoblotted using antibody to either myosin IIA (MYH9) or myosin IIB (MYH10). In (A), (B), (D) and (E), columns represent population means. Error bars show standard error of the mean. Data points represent values of each metric.
for one cell. Comparisons between control and treated cells were made using the Mann-Whitney test. *, p<0.05; **, p<0.01; n.s., not significant. In (C) and (F), data show overall fraction of contact guided cells, and bars show 95% confidence intervals of the fractions. Comparisons between fractions of treated and control cells were analyzed using the two-population proportion z test. *, p<0.05; n.s., not significant.
Figure 8-9. The roles of actomyosin contractility, Cdc42, and Rac1 in the migration and contact guidance of HT1080 fibrosarcoma cells. HT1080 cells were treated with blebbistatin (50 µM), ML141 (20 µM), or the appropriate vehicle control. (A) Average speed and (B) persistence were quantified in the 20 µm-wide feeder microchannels for prescribed pharmacological interventions. In (A) and (B), columns represent population means, and error bars show standard error of the mean. Data points represent values of each metric for one cell. Comparisons between
control and treated cells were made using the Mann-Whitney test. n.s., not significant; ****, p<0.0001. (C,D) Fraction of contact guided cells during the decision making process. In (C) and (D), data show overall fraction of contact guided cells, and bars show 95% confidence intervals of the fractions. Comparisons between fractions of treated and control cells were analyzed using the two-population proportion z test. **, p<0.01.
To confirm that actomyosin contractility is a negative regulator of contact guidance in 20 μm-wide feeder channels for MDA-MB-231 cells, we knocked down non-muscle myosin isoforms IIA and IIB via siRNA (Fig. 8-8G; Fig. 8-10). Of the three non-muscle myosin II isoforms, MDA-MB-231 cells express myosin IIA and myosin IIB (Betapudi et al., 2006). Individual knockdown of myosin IIA or myosin IIB (Fig. 8-10D) did not affect cell contact guidance, speed or persistence compared to matched controls (Fig. 8-10E-G) at two siRNA doses (0.06 or 0.12 pmol siRNA/ml transfection medium). In contrast, cells depleted of both myosin isoforms via siRNA (0.06 pmol siRNA per isoform/ml transfection medium) (Fig. 8-8G) migrated at slower speed but similar persistence compared to cells treated with non-targeting Ctrl siRNA (Fig. 8-8A,B). Moreover, myosin IIA- and IIB-knockdown cells were significantly less contact guided relative to controls (Fig. 8-8C). Taken together, we conclude that the crosslinking of actin to myosin is essential for contact guidance for this cell line, with compensation pathways available when this linkage is not fully abrogated.
Figure 8-10. Individual knockdown of non-muscle myosin II isoforms, MIIA and MIIB, does not modulate contact guidance of MDA-MB-231 cells. (a) MDA-MB-231 cells were transfected with Ctrl siRNA or siRNA targeting myosin IIA (MYH9) or myosin IIB (MYH10) using either 0.06 or 0.12 pmol siRNA per ml of transfection medium. Knockdown was confirmed via Western blot, with actin as loading control. (b) Effect of knockdown of myosin II isoforms using two siRNA concentrations on the fraction of contact guided cells. Columns show fraction of contact guided cells, and bars show 95% confidence intervals of the fractions. Comparisons between proportions of treated and control cells were made using the two-population proportion z test. (c)
Average speeds and (d) persistence ratios of cells migrating inside 20 µm-wide feeder microchannels were quantified for the prescribed molecular interventions. In (c), (d), columns represent population means, and error bars show standard error of the mean. Data points represent values of each metric for one cell. Comparisons between matched control and treated cells were assessed by Kruskal-Wallis test with post-hoc Dunn’s multiple comparisons test. *, p<0.05; **, p<0.01.
8.3.5. Inhibition of Cdc42 increases contact guided-mediated decision making in MDA-MB-231 and HT1080 cells. In light of our data showing an association between cell polarization and contact guidance of MDA-MB-231 cells (Fig. 8-5) and because RhoGTPase Cdc42 is critical to cell polarization (Wedlich-Soldner et al., 2003), we examined its contribution to contact guidance. MDA-MB-231 cell treatment with the Cdc42 inhibitor ML141 did not affect migration speed (Fig. 8-11A) but increased cell persistence (Fig. 8-11B) in the straight $20 \, \mu$m-wide feeder channels. This pharmacological intervention tended to increase the fraction of contact guided MDA-MB-231 cells at bifurcations (Fig. 8-11C). It is noteworthy that HT1080 fibrosarcoma cells, which display moderately lower baseline levels of contact guidance than MDA-MB-231 cells, became significantly more contact guided upon Cdc42 inhibition via ML141 (Fig. 8-9F). Interestingly, inhibition of Cdc42 failed to affect MDA-MB-231 cell speed and persistence inside narrow ($3 \, \mu$m in width) feeder channels (Fig. 8-11E-F) as well as their decision making (Fig. 8-11D).
Figure 8-11. Inhibition of Cdc42 increases contact guidance-mediated decision making in MDA-MB-231 cells. MDA-MB-231 cells were treated with ML141 (20 µM) or vehicle control. In separate experiments, cells were transfected with Ctrl siRNA or siRNA targeting Cdc42. (A) Average speeds and (B) persistence quantified in the 20 µm-wide feeder microchannels for prescribed pharmacological and molecular interventions. (C) Fraction of contact guided cells during the decision making process. (D) Average migration speed and (E) persistence of cells treated with ML141 (20 µM) or vehicle control in 3 µm-wide feeder channels. (F) Fraction of cells migrating in 3 µm-wide feeder channels that enter 20 µm-wide branch channels in the presence or absence of ML141. (G) Knockdown of Cdc42 was confirmed via Western blot, with actin as loading control. In (A), (B), (D) and (E), columns represent population means. Error bars show standard error of the mean. Data points represent values of each metric for one cell. Comparisons between control and treated cells were made using the Mann-Whitney test. *,
p<0.05; n.s., not significant. In (C) and (F), data show overall fraction of contact guided cells, and bars show 95% confidence intervals of the fractions. Comparisons between fractions of treated and control cells were analyzed using the two-population proportion z test. *, p<0.05; n.s., not significant.
The impact of Cdc42 on the decision making of cells migrating inside 20 µm-wide feeder microchannels was further examined by transiently knocking down Cdc42 in MDA-MB-231 cells. Knockdown was confirmed via Western blot (Fig. 8-11G). Cdc42-knockdown cells migrated slightly slower but with the same persistence as cells transfected with non-targeting Ctrl siRNA (Fig. 8-11A,B). Importantly, a significantly larger fraction of Cdc42-KD cells were contact guided at the bifurcation when compared to control cells (Fig. 8-11C). To our knowledge, this is the first study showing how Cdc42 affects migration in microchannels. Taken together, these data suggest that while interfering with Cdc42 function has little impact on cell speed and persistence in 20 µm-wide microchannels, it promotes contact guidance-mediated decision making.

We next examined the role of Rac1 in decision making at bifurcations. Rac1 is a RhoGTPase involved in lamellipodia formation (Rottner et al., 2011). Inhibition or knockdown of Rac1 in MDA-MB-231 cells did not affect migration speed or persistence in 20 µm-wide feeder channels but elicited a moderate increase in contact guidance (Fig. 8-12). Similar effects were seen in HT1080 cells, where inhibition of Rac1 caused a modest but not statistically significant increase in the fraction of cells contact guided at the bifurcation (Fig 8-9G-I).
**Figure 8-12. The effect of inhibition of Rac1 on MDA-MB-231 cell contact guidance-mediated decision making at bifurcations.** MDA-MB-231 cells were treated with NSC23766 (10 µM) or vehicle control. In separate experiments, cells were transfected with Ctrl siRNA or siRNA targeting Rac1. (A) Average speeds and (B) persistence quantified in the 20 µm-wide feeder microchannels for prescribed pharmacological and molecular interventions. (C) Fraction of contact guided cells during the decision making process. (D) Knockdown of Rac1 was confirmed via Western blot, with actin as loading control. In (A) and (B), columns represent population means, and error bars show standard error of the mean. Data points represent values of each metric for one cell. Comparisons between control and treated cells were made using the Mann-Whitney test. n.s., not significant. In (C), data show overall fraction of contact guided cells, and bars show 95% confidence intervals of the fractions. Comparisons between fractions of treated and control cells were analyzed using the two-population proportion z test.
8.4. Discussion

While studies have elucidated how cells switch between mesenchymal and amoeboid migration modes to navigate fibrillar collagen-rich tissue (Friedl et al., 2010), there is no conceptual framework for understanding cell decision making through tunnel-like anisotropic microenvironments that are prevalent in vivo (Alexander et al., 2008; Alexander et al., 2013; Friedl et al., 2011; Weigelin et al., 2012). We discovered that different types of confinement (2D versus 3D-like confinement or partial versus full confinement) exert distinct effects on MDA-MB-231 cell decision making at bifurcations. MDA-MB-231 cells migrating from feeder regions in which they fully explore the microenvironment favor entry into wider branches. This is the case for cells migrating on laterally confined 2D printed lines as well as inside narrow microchannels (i.e., full confinement). In contrast, repression of cell lamellipodia at bifurcation, which can be achieved in wider feeder channels due to contact guidance and migration along a single side wall, results in pore size-independent decisions at bifurcations provided that the cells are fully polarized. It is noteworthy that cell polarization requires a critical length scale (Fig. 8-5). While migration of 3T3 Mouse Swiss Albino embryo fibroblasts along edges of micromachined grooves has been observed (Kaiser et al., 2006), this is, to our knowledge, the first observation of the direct impact of contact guidance on cell decision making. Taken together, MDA-MB-231 cell decision making at bifurcations is dependent on the dimensionality (2D versus 3D) as well as the lateral and axial length scales of feeder channels. The novelty of these findings illustrates the power of using microfabrication techniques as model systems for studying cell migration. It is noteworthy that such a fine control of microenvironmental topography to elucidate length
scale-dependent decisions is not possible in fibroblast-remodeled ECM gels (Shieh et al., 2011) or along bulk-aligned collagen fibers (Provenzano et al., 2008).

From the molecular perspective, persistent elongation and orientation along a single side wall in wide feeder channels (i.e., contact guidance) requires β1 integrin expression in both MDA-MB-231 and HT1080 cells. Moreover, inhibition of cell contractility via blebbistatin suppresses contact guidance in these cell lines. Importantly, dual knockdown of myosin IIA and IIB is required for suppressing contact guidance of MDA-MB-231 cells. It is noteworthy that individual knockdown of myosin II isoforms failed to alter the fraction of contact guided cells, suggesting a requirement for universal abrogation of actomyosin contractility and the existence of a compensatory mechanism between myosin IIA and IIB. Along these lines, knockdown of RhoA did not affect MDA-MB-231 cell contact guidance (Fig. 8-13). We thus postulate that concomitant inhibition of RhoA- and myosin light chain kinase (MLCK)-driven activity is needed to repress contact guidance in this cell line. This is substantiated by our findings showing that simultaneous inhibition of RhoA/ROCK via Y-27632 and MLCK via ML-7 is needed to suppress the fraction of contact guided MDA-MB-231 cells (Fig. 8-13). On other hand, contact guidance was promoted in MDA-MB-231 and HT1080 cells when Cdc42 function was disrupted. Consistent with the finding that Cdc42 inhibition increases contact guidance and migration persistence through complex physical spaces, knockdown of Cdc42 has been reported to increase MDA-MB-231 cell migration and invasion in transwell assays (Zuo et al., 2012).
Figure 8-13. Concurrent inhibition of both RhoA/ROCK and MLCK is required for modulation of contact guidance in MDA-MB-231 cells. (A) MDA-MB-231 cells were transfected with Ctrl siRNA or siRNA targeting RhoA. Knockdown was confirmed via Western blot using actin as loading control. (B) Effect of RhoA knockdown or simultaneous treatment with ML-7 (20 µM) and Y-27632 (30 µM) on the fraction of contact guided MDA-MB-231 cells. Columns show fraction of contact guided cells, and bars show 95% confidence intervals of the fractions. Comparisons between proportions of treated and control cells were made using the two-population proportion z test. *, p<0.05.
Myosin II is an important regulator of cell shape and mechanosensing (Frey et al., 2006) in complex topographies. Suppression of secondary protrusions in contact guided cells may be due to the high energy barrier required to break actin bundles aligned along the direction of elongation (Mahmud et al., 2009) or increases in membrane tension in elongated cells (Houk et al., 2012). Myosin II is required to minimize cell curvature (Elliott et al., 2015), suggesting that interfering with actomyosin contractility may inhibit contact guidance by lowering the threshold at which new protrusions could be generated from the cell body. In light of our data showing that actomyosin contractility and β1 integrin are critical to contact guidance, we cannot rule out a potential interplay between these two molecular constituents. This scenario is supported by prior work showing that blebbistatin reduces the size of focal adhesions in 2D (Pasapera et al., 2010).

This study highlights the need for characterizing and standardizing the precise physical microenvironmental features in cell migration studies. Such standardization has recently helped explain seemingly contradictory results in 3D ECM gels (Wolf et al., 2013). Confinement to printed lines has been put forth as a model of 3D migration (Doyle et al., 2009), but it is unlikely that this adhesion-dependent mode of migration recapitulates adhesion-independent 3D migration modalities observed by us (Balzer et al., 2012) and others (Tozluoglu et al., 2013; Liu et al., 2015; Bergert et al., 2015). Importantly, distinct decision making outcomes are noted when cells are confined within microchannels versus on laterally confining lines. It is thus imperative to define and decipher how microtopology, particularly on the length scale of interstitial spaces, can potentiate and impact cell migration.
In summary, the alignment and migration of cells along cell-scale topographical features have important applications pertinent to cancer metastasis, where cells migrate through tunnel-like confining \textit{in vivo} microenvironments (Alexander et al., 2008; Alexander et al., 2013; Gritsenko et al., 2012; Naumov et al., 1999; Weigelin et al., 2012); in cancer immunology, in which immune cell infiltration of tumors is hindered by high peritumoral ECM concentrations (Salmon et al., 2012; Hartmann et al., 2014); and in tissue engineering, where there is a need to precisely and anisotropically place and align cells in specific tissue structures (Engelmayer et al., 2008; Bian et al., 2009; Nawroth et al., 2012). Tools to study functional cell response to topographic features will contribute to discoveries in these fields.
Chapter 9

FUTURE DIRECTIONS AND CONCLUDING REMARKS

9.1. Introduction

Bioengineering assays to study cell migration have revealed a number of novel cell migration mechanisms (Liu et al., 2015; Stroka et al., 2014b) and elucidated the incredible plasticity of cell migration (Friedl et al., 2010). While these studies offer important biological insights and point to possible microenvironmental and genetic targets to control cell migration, their translation to clinical settings has thus far been limited. In this chapter, we discuss early translational applications that the study of mechanobiology and cell migration in microfluidic devices has engendered. We then describe how the Y-shaped microchannels used in Chapter 8 could be used to assess the metastatic propensity of different breast cancers, giving suggestions for the development of this application. We conclude with some thoughts on the future of microfluidic microchannel migration assays.

9.2. Translational Applications of Studying Cell Behavior in Confinement

While years of studies in biology labs have elucidated important genetic underpinnings of cancer and other aspects of human health, exploitation of cell migration in physiological environments, with appreciation of the effects of confinement and cell
mechanosensing, to treat disease is in its infancy. Below, we summarize several early translational applications that the study of cell behavior from a physical perspective have enabled.

9.2.1. **Anti-metastatic effects of modulating the tumor microenvironment.** The response of tumor cells to the physical microenvironment may be exploitable for treating cancer and other diseases. For example, glioblastoma multiforme (GBM) cells preferentially migrate along white matter tracts and blood vessels to invade the brain, making surgical resection difficult (Cuddapah et al., 2014). Jain and colleagues took advantage of GBM invasion along topographical cues to draw cells away from a GBM tumor established in a mouse model (Jain et al., 2014). An axon guide was filled with an aligned nanofiber membrane and implanted adjacent to a GBM tumor, and U87MG-eGFP cells preferentially migrated through the guide to significantly reduce the tumor volume in the brain (Jain et al., 2014). Importantly, the aligned topography of the nanofiber membrane was crucial for this result, as empty axon guides or conduits filled with a smooth (instead of topographically patterned) film did not lead to the same degree of tumor reduction (Jain et al., 2014). The conduit could be paired with a cyclopamine-conjugated collagen hydrogel to selectively cause apoptosis in cells migrating into the conduit and expressing the sonic hedgehog (Shh) pathway, which is overexpressed in most high-grade brain tumors (Jain et al., 2014). Along these lines, exploitation of mechnosensitive pathways used by cells to sense the physical microenvironment may target cells in regions of dysplasia. A recent study found that patient-derived GBM cells lack the sensitivity to substrate stiffness (e.g., cell rounding and impaired migration on
soft substrates) that is seen in U373-MG GBM cell line, making these cells highly migratory across the stiffness range seen in the brain (Wong et al., 2015). When these patient-derived cells were transfected with constitutively active RhoA, ROCK, or MLCK, contractility increased and cells regained stiffness sensitivity, presumably due to an increased ability to deform the matrix and sense its stiffness (Wong et al., 2015). When cells expressing constitutively active RhoA were orthotopically implanted in a mouse xenograft model, survival significantly increases compared to the control case, with a reduction in tumor cell invasion in the brain and tumor size (Wong et al., 2015). In summary, exploitation of tumor mechanosensitive pathways and topography-mediated functional behaviors represents an underexplored therapeutic pathway.

9.2.2. Elucidation of single-cell vs. population-level characteristics. As microfluidic technologies combine real-time, single-cell resolution with population-scale throughput, these assays will help elucidate the causes and consequences of heterogeneity in cell populations. Recent work with confined migration assays has illustrated the utility of such an approach. Confinement of HCT-116 human colon carcinoma and MCF-7 human breast carcinoma cells in 10 µm x 10 µm PDMS microchannels revealed that the migration speed of daughter cells does not correlate with the speed of the mother cell, even though the population-level speed of mother and daughter cell populations was identical (Yan et al., 2014). Collection of MDA-MB-231 cells that successfully transmigrated through confining microchannels revealed that highly chemotactic cells had a more elongated morphology than non-chemotactic cells and, after standard subculture, retained their migratory phenotype, and these cells expressed higher levels of
GTPase RhoC and p38γ (Chen et al., 2015). This result suggests that a microchannel device could be used as a “filter” to select the most migratory cells from a given population. Such studies, performed using genetically identical cells, illustrate the unknown determinants of cell migration at the single-cell level, where cell genome should be identical. Research building on these results will help determine whether and how to target those cells most responsible for metastatic spread.

9.2.3. **High-throughput drug screening assays in physiologically-relevant microenvironments.** Combination of microfluidics and confined migration assays will enable high-throughput drug screening in physiologically-relevant microenvironmets. For example, single-device screening of small molecule inhibitors demonstrated enhanced migration of Taxol-resistant SUM-159 cells through 10 x 10 µm² microchannels upon exposure to Axitinib and MMP-9 inhibitor 444278, while the same cells displayed reduced migration when treated with linifanib (Zhang et al., 2014). Such devices allow simultaneous, real-time monitoring of physiologically-relevant cell responses to inhibitors, require low numbers of cells (thousands to tens of thousands), and are compatible with samples from primary tumors (Zhang et al., 2015). Combination of such as approach with LFI imaging would greatly speed drug discovery and efficacy efforts.

9.2.4. **Point-of-care microfluidic devices using whole blood from patients.** The small number of cells used in microfluidic devices make them especially promising for point-of-care applications. Thus far, these applications, at least in the context of cell migration,
have been explored using immune cells. For example, neutrophils isolated from burn patients migrate at slower speeds through PDMS microchannels than those from healthy volunteers, and speed correlated to the size of the burn injury (Butler et al., 2010). Later iterations of this device were able to handle unprocessed blood at very small volumes (~2 µl) to demonstrate decreased neutrophil migration up an fMLP gradient after a burn injury, with a 75% reduction in velocity and a 50% reduction in migration toward the chemoattractant (Hoang et al., 2013). Neutrophil speed was still impaired at 2 weeks-post burn, but migration directionality was recovered (Hoang et al., 2013). By three-weeks post-burn, neutrophil recruitment, velocity, and directionality approached normal values (Hoang et al., 2013). A similar device was able to distinguish between burn patients developing sepsis and those not developing sepsis based on the paths taken by neutrophils migrating in a microfluidic device with maze-like microchannels; specifically, spontaneous migration in the absence of a chemoattractant gradient occurred primarily in patients developing sepsis (Jones et al., 2014). Importantly, the “score” generated by this device distinguished between patients developing sepsis and not developing sepsis up to two days before clinical diagnosis, suggesting that it could be used as an early aid in determining antibiotic dosing (Jones et al., 2014). Such devices may also help discover treatments that rapidly recover proper neutrophil function and migration after burns. For example, the migration efficiency of neutrophils in a microfluidic device correlates with survival in a rat burn model, with neutrophil function in vitro and in vivo largely restored by treatment with resolvin RvD2 following the burn (Kurihara et al., 2013). Resolvin treatment also made burned rats less susceptible to microbial insult (Kurihara et al., 2013). Similar studies with microfluidic channels have
suggested aberrant migration of fibroblasts with a mutation in the TOR1A gene that underlies DYT1 dystonia (Nery et al., 2014), examined microglial chemotaxis up a soluble amyloid-β gradient in an Alzheimer’s model (Cho et al., 2013), and elucidated how neutrophil retrotaxis prevents detrimental neutrophil accumulation at sites eliciting an immune response (Hamza et al., 2015).

9.3. A Cell Decision Making Device for Assessing Metastatic Propensity

A current challenge in cancer treatment is assessing the aggressiveness of a given cancer at diagnosis and tailoring a treatment plan accordingly. The microfluidic device containing Y-shaped microchannels that is discussed in detail in Chapter 8 has shown promise in distinguishing between aggressive and non-aggressive breast cancer cell lines. Preliminary experiments with this device for the purpose of gauging metastatic propensity are discussed below, and suggestions for future experiments are given.

9.3.1. Microfluidic device design for metastatic propensity assay. The microfluidic device described in Chapter 8 was modified. Microchannels were 20 μm wide, 10 μm tall, and bifurcated to 10 μm-wide or 3 μm-wide branch channels at a distance of 265 μm from the channel entrance. The design was also changed to enhance throughput by increasing the number of Y-shaped microchannels contained on each device. In brief, the device was lengthened and the angle at the bifurcation was increased from 45° to 65° from the horizontal. With these adjustments, each device contained 243 channels (as opposed to 20 channels in the earlier design). Additionally, the width of the cell seeding line oriented perpendicularly to the microchannel entrances was decreased in width from 400 μm to 150 μm to give cells better access to the microchannel entrances. With these
adjustments, 50,000 cells are seeded per experiment. This represents ~1/50 the volume of a typical resected tumor.

9.3.2. Characterization of migratory and non-migratory cells in human breast cancer cell lines. Human breast cancer cells were seeded at the microchannel entrances as described in Chapter 8, and their migration was monitored using time lapse phase contrast microscopy over approximately 24 h. The total number of cells entering the microchannels over this time was counted. Of this population, cells were divided into two groups: migratory and non-migratory. Migratory cells were those which made a decision at the bifurcation and migrated into a branch channel (Fig. 9-1A). Non-migratory cells did not enter either of the branch channels over the course of the experiment (Fig. 9-1B).

We observed that this binary grouping led to divergent cell phenotypes in MDA-MB-231 cells. Migratory MDA-MB-231 cells were significantly faster than non-migratory cells, migrated more persistently, and had lower circularity (that is, were more elongated) (Fig. 9-1C,D,F). Furthermore, migratory cells were more aligned to the microchannel walls, as judged by decreased variance in the fit elliptical angle of migratory cells (Fig. 9-1E). These results suggest that cell spontaneously segregate into a subpopulation that moves at high speed, persistence, and contact guidance after entering the microchannels.
Figure 9-1. Spontaneous segregation of MDA-MB-231 cells into migratory and non-migratory subpopulations in Y-shaped microchannels. MDA-MB-231 cells migrating within microchannels were classified into two groups: (A) migratory cells, which made a decision and entered a branch microchannel during an experiments; and (B) non-migratory cells, which remained in the feeder section of the microchannel. Migratory cells had significantly higher (C) speed and (D) chemotactic index (persistence ratio) than non-migratory cells. (E) The standard deviation of fit elliptical angles of migratory cells was also lower than for non-migratory cells, indicating contact guidance. (F) Migratory cells further had lower circularity than non-migratory cells and were more elongated in the microchannels. *, p<0.05 by two-tailed t test.
9.3.3. Correlation of migration in the microfluidic device with metastatic propensity.

A panel of human breast cell lines was assayed in the microfluidic device, and the percentage of migratory cells was calculated. Percentages were calculated as the average of migratory cell percentages over several experiments. While non-tumorigenic MCF10A cells and non-metastatic MCF7 and MDA-MB-468 cells had low (<5%) percentages of migratory cells, metastatic cell lines had high percentages of migratory cells (>10%) (Table 9-1). Furthermore, treatment of triple negative breast cancer (TNBC) cell lines with LY294002, and inhibitor of PI3K, had differential effects on the percentage of migratory cells in the device, even those these cell lines would all be classified together using current diagnostic standards (Table 9-2). This proof-of-concept experiment suggests the promise of this device for screening of both metastatic propensity and personalized drug therapy.
Table 9-1. Metastatic propensity of human breast cancer cell lines correlates with the percentage of migratory cells in Y-shaped microchannels. A panel of human breast cell lines was assayed in the Y-shaped microchannel device, and the percentage of migratory cells (cells reaching the branch channels) was enumerated. Non-metastatic cell lines with low tumor-initiating cell counts had low migration in the device (<5% migratory cells), while more metastatic cell lines with more tumor-initiating cells had a higher percentage of migratory cells (>10%).

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Metastatic</th>
<th>Tumor-Initiating Cells</th>
<th>Control % Migratory Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF10A</td>
<td>No</td>
<td>-</td>
<td>0%</td>
</tr>
<tr>
<td>MCF-7</td>
<td>No</td>
<td>-</td>
<td>0%</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>No</td>
<td>+</td>
<td>1 ± 1%</td>
</tr>
<tr>
<td>K-Ras/OE3CN-KD MCF10A</td>
<td>Yes</td>
<td>+++</td>
<td>20%</td>
</tr>
<tr>
<td>MDA-MB-436</td>
<td>Yes</td>
<td>+++</td>
<td>13 ± 7%</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>Yes</td>
<td>+++</td>
<td>22 ± 3%</td>
</tr>
<tr>
<td>Bi-549</td>
<td>Yes</td>
<td>+++</td>
<td>32 ± 8%</td>
</tr>
<tr>
<td>Hs578i</td>
<td>Yes</td>
<td>+++</td>
<td>20 ± 7%</td>
</tr>
</tbody>
</table>
Table 9-2. The migration of triple negative breast cancer cells in Y-shaped microchannels is differentially impacted by PI3K inhibition. Treatment of breast cancer cell lines with the PI3K inhibitor LY294002 led to an increase in the percentage of migratory MDA-MB-231 cells and a decrease in the percentage of migratory Bt-549 cells. The migration of MDA-MB-436 and Hs578t cells was unchanged by inhibition of PI3K signaling. These cell lines are all triple negative breast cancer (TNBC) cells.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Metastatic</th>
<th>Tumor-Initiating Cells</th>
<th>Control % Migratory Cells</th>
<th>PI3K Inhibition % Migratory Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-436</td>
<td>Yes</td>
<td>+++</td>
<td>13 ± 7%</td>
<td>18 ± 7%</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>Yes</td>
<td>+++</td>
<td>22 ± 3%</td>
<td>34 ± 8%</td>
</tr>
<tr>
<td>Bt-549</td>
<td>Yes</td>
<td>+++</td>
<td>32 ± 6%</td>
<td>19 ± 1%</td>
</tr>
<tr>
<td>Hs578t</td>
<td>Yes</td>
<td>+++</td>
<td>20 ± 7%</td>
<td>15 ± 2%</td>
</tr>
</tbody>
</table>
9.3.4. Isolation of migratory MDA-MB-231 cells. Migratory MDA-MB-231 cells that had successfully transmigrated through the microchannels were extracted from the device via selective trypsinization of cells in channel connecting to the branch lines. Images of the microchannels before (Fig. 9-2A, left panel) and after (Fig. 9-2A, right panel) removal of migratory cells are shown in Figure 9-2. With this method, cells within microchannels and in the cell seeding area at the microchannel entrances remain in the device, while migratory cells that have exited the microchannels are removed from the device and collected.

Isolated migratory MDA-MB-231 cells and cells removed from the cell seeding area were mixed with Matrigel and injected orthotopically into mice (~300 cells/condition). While both mice developed intramammary tumors 8 weeks post-injection, only the mouse injected with migratory cells developed micrometastases in the lung (Fig. 9-2B). This suggests that the cells scoring as migratory in the device are those cells most responsible for metastasis.
Figure 9-2. Isolated migratory MDA-MB-231 cells are more likely to cause micrometastases following orthotopic injection in mice. (A) MDA-MB-231 cells were seeded at the entrances of Y-shaped microchannels and migrated through the channels overnight. The cells that had transmigrated through the channels (left panel) were trypsinized, removed from the device, and collected, leaving non-migratory cells in the feeder microchannels and cell seeding area (right panel). (B) Migratory cells and control cells removed from the cell seeding area of the device were orthotopically injected in the mouse mammary fat pad (~300 cells/condition). Both cell populations formed intramammary tumors by 8 weeks post-injection. However, only the mouse injected with migratory cells developed micrometastases in the lung.
9.3.5. Suggestions for future experiments: Y-microchannel metastatic propensity assay. To ultimately develop the Y-microchannel assay for use as a clinical tool that can quickly (~16 h) gauge the likelihood of a patient’s cancer to metastasize, the migration of cells in the device must be correlated with clinical outcomes. As an initial step, a panel of cell lines should be run in the device, with the percentage of migratory cells quantified. The percentage of migratory cells over time should also be noted to determine the optimal time to run the assay, and to determine whether this parameter is cell line- or cancer-type-dependent. These cell lines should be simultaneously injected orthotopically in immunocompromised mice, and the rate of tumor growth, metastasis, and survival of the mice should be monitored. This will allow a database to be built, from which stronger correlations between cell behavior in the microfluidic device and functional outcome can be made.

Next, the device should be tested with primary cell lines from patients with known outcomes. For example, a bank of transplantable tumor grafts has been established for breast cancer patients (DeRose et al., 2011). If the percentage of migratory cells measured in the device correlates with metastasis and/or survival from this cell bank, the clinical case for using the device would be much stronger. If the device can eventually be shown to blindly predict patient outcome upon assay immediately after surgery but before patient outcome is known, it would be shown to be even more useful.

Finally, important mechanistic data could be collected by isolating and characterizing migratory cells. These cells may be characterized by their likelihood to cause metastasis in mice, their growth rate and ability to grow upon limiting dilution in tumorsphere assays, or by single cell genomics and proteomics. Such studies would
delineate underlying differences between migratory and nonmigratory cells and could also help indicate whether the migratory cells isolated have stem-like properties. For example, this study could help determine whether rapidly migrating tumor cells are cancer stem cells, or whether stem-like cells compromise migration for self-renewal. Additionally, “-omics” techniques would reveal whether genetic or epigenetic heterogeneity in the population is responsible for functional differences between migratory and nonmigratory cells, or whether their migration is simply a function of access to the microchannel entrances. If migratory cells are found to be responsible for metastasis and can be isolated and cultured, they could be tested for response to pharmacological agents to target those cells most likely to cause metastatic spread.

9.4. Outlook and Perspectives: The Future of Microchannel Migration

Assays

In vitro models of physical confinement have revealed numerous mechanisms underlying tumor cell migration. Going forward, microfluidic assays should help answer open questions about tumor progression and metastasis and be integrated into point-of-care diagnostic devices. We have detailed how these assays may be used to answer fundamental questions about cancer biology, how they may be applied to direct cell migration in vivo through physical mechanisms, and how the low cell count required for microfluidic assays makes them promising candidates for real-time, personalized diagnostics. Importantly, because microfluidics enables single-cell sampling at the population level, these assays may help elucidate the role of genetic heterogeneity in cell behavior. Cancer cells have increased genetic and epigenetic instability (Potapova et al.,
2013), leading to genetic heterogeneity from even single-cell derived clones. In the assays described in this dissertation, this heterogeneity may be picked up in the observed spread in cell speed and persistence, although we did not run any genetic tests. Future work could combine some functional cell behavior (for example, cell speed or division time) with emerging single-cell sequencing technologies to show how heterogeneity develops in a population of tumor cells over time, and how this heterogeneity affects cell function.

Ultimately, microchannel-like features should be integrated on “organ-on-a-chip” devices to account for the architecture of tissue structures in in vitro models of tumor growth and metastasis. Accommodating for the diverse mechanisms available to confined tumor cells, the interaction of tumor cells with associated stromal cells (Sharma et al., 2012), and environmental conditions such as hypoxia (Zhang et al., 2015) and the interplay between chemotaxis and contact inhibition (Lin et al., 2015) at the bench stage of drug discovery will increase the clinical success rate new of anti-cancer drugs.

Finally, we envision a scenario where the Y-shaped microfluidic channels described above can be used at clinics to inform rapid decisions about the aggressiveness of an individual cancer. Ideally, cells from a portion of a biopsy or surgically resected tumor could be seeded in the migration device and monitored over 16-24 h. Real-time imaging of cells using LFI would make this assay affordable at the clinical level, as it could be operated in a standard cell culture incubator using an imaging platform costing (at most) several thousand instead of tens of thousands of dollars. The outcome of this assay could inform a clinician on how aggressive treatment should be, improving either survival or patient quality of life. Furthermore, microfluidics make possible the testing of
primary cancer cell migration under a variety of pharmacological or biological agents. The device could thus be used to test various treatments on patient samples, either initially or after isolation and expansion of the highly migratory cell subpopulation. We are hopeful that translational uses of the confined migration assay and LFI imaging technique detailed in this thesis could have a positive impact on cancer treatment.


antigen capture are antagonistic processes coupled by myosin II in dendritic cells.

*Nat Commun* **6**: 7526. doi: 10.1038/ncomms8526.


CURRICULUM VITAE

Colin D. Paul was born in Monterey, California on August 16, 1987 to Chris and Anne Paul. He has a twin brother, Ryan, and a younger brother, Patrick. Colin graduated as a National Merit Scholar from Union High School in Tulsa, Oklahoma in 2006 before enrolling at the University of Arkansas (Fayetteville, Arkansas). There, he majored in Chemical Engineering and Physics and received a minor in Mathematics. Colin performed undergraduate research under Dr. Jamie Hestekin at Arkansas, studying the removal of butanol from simulated fermentation broths using pervaporation through polydimethylsiloxane (PDMS) and mixed matrix PDMS/zeolite membranes. He presented his work at the 2009 Mid-America Regional Conference of the American Institute of Chemical Engineers, winning 1st place in the Undergraduate Student Paper Competition. Colin also led the research arm of a design team working on improved pretreatment for shipboard reverse osmosis systems to a 1st place finish in the 2010 WERC Environmental Design Competition.

Following graduation from the University of Arkansas, Colin enrolled in the Ph.D. program in the Department of Chemical and Biomolecular Engineering at Johns Hopkins University (JHU) in Baltimore, Maryland. His wife, Michelle Shepherd Paul, joined him in Baltimore in 2011 after completing her degree in Chemical Engineering at the University of Arkansas. Colin accepted a predoctoral research assistantship in the lab of Dr. Konstantinos Konstantopoulos in January 2011 and was awarded a National Science Foundation Graduate Research Fellowship that year to support his graduate work. He was also accepted Nano-Bio Graduate Training Program run by the JHU
Institute for NanoBioTechnology (INBT). His research focused on tumor cell migration in microchannels that recapitulated tunnel-like migration tracks found in the human body. After helping to find that inhibitors of actomyosin contractility did not affect tumor cell migration in highly confining microchannels (FASEB J, 2012), he worked with Dr. Phrabha Raman to design a device to measure traction force exertion during confined migration by incorporating deflectable microposts within confining microchannels (Lab Chip, 2013). They found that measured traction forces are significantly reduced in narrow channels vs. wide channels. Additionally, force exertion in narrow channels was not affected upon treatment with pharmacological agents that activate or inhibit contractility. This work was awarded a Biomedical Engineering Society Graduate Design and Research Award in 2013.

Following examination of cell migration in straight microchannels, Colin and his colleagues worked to unravel how cells make decisions in more complex microenvironments. Colin designed and fabricated bifurcating microchannels to show that, in environments wider than the cell body, MDA-MB-231 breast adenocarcinoma cells are contact guided along one microchannel wall and migrate into contiguous “branch” channels at a bifurcation, regardless of the branch microchannel width. When cells were confined in channels narrower than the cell body or migrate on microcontact printed patterns (instead of within three-dimensional channels), they instead preferentially choose wide branch regions at a bifurcation. In wide microchannels, inhibiting contractility via siRNA-mediated knockdown of myosin isoforms or treatment with blebbistatin reduced the propensity of cells to persistently migrate along a topographical feature. Inhibiting the Rho GTPase Cdc42 (considered responsible for
filopodia formation) enhanced cell contact guidance along a topographical feature. Similar results were obtained with HT1080 fibrosarcoma cells. This work was performed in collaboration with Daniel Shea. Microfluidic devices incorporating microchannels with complex designs have further shown promise in predicting the metastatic propensity of different adherent and primary cell lines (U.S. Provisional Patent P12511-01, 2013). Specifically, the ability of cell lines to traverse complex topographies in vitro correlated with the metastatic propensity of the cell line in mice. Furthermore, in initial tests, MDA-MB-231 cells that had successfully transmigrated through Y-shaped channels (termed “migratory cells”) were more likely to cause metastasis following orthotopic transplantation in mice than cells from the general MDA-MB-231 cell population.

Colin also worked with collaborators from imec (Leuven, Belgium) to translate a novel lens-free imaging (LFI) platform for use in cell migration assays. The work was initiated through JHU INBT International Research Experience for Students grants that Colin received in 2012 and 2013. Initial experiments were performed in the summers of 2012 and 2013, when Colin spent time at imec and worked with Evelien Mathieu to engineer microfluidic devices that could be imaged using LFI. The LFI system offers a 50-fold larger field of view compared to a standard phase contrast microscope at approximately 1/50 of the cost. Evelien visited the Konstantopoulos lab in 2015 to benchmark the LFI platform against phase contrast time lapse microscopy for a number of single cell motility assays. Furthermore, Colin and Evelien used LFI to show how different types of physical confinement drive diverse cell migration responses in metastatic MDA-MB-231 breast cancer cells and non-metastatic MCF7 breast cancer cells. For example, “one-dimensional” confinement to microcontact printed lines leads to
an increase in cell migration efficiency compared to migration on a two-dimensional surface, but it is not equivalent to three-dimensional confinement in microchannels, where the most efficient migration was observed. Ideally, LFI could be combined with a metastatic propensity migration assay to predict the aggressiveness of an individual cancer at low cost and high throughput.

In addition to research, Colin greatly enjoyed teaching and mentoring during his time at Johns Hopkins. He was a teaching assistant for Transport Phenomena I with Dr. Konstantopoulos in 2012 and 2013, and he led a module on microfabrication for the 2013 NanoBioTechnology Lab course. He mentored three undergraduate researchers at Johns Hopkins, each of whom contributed substantially to the contact guidance project. Colin also mentored Baltimore-area high school students in his work with Thread, a non-profit committed to ensuring educational opportunities for underprivileged students. Colin’s future research goals are in in vitro models of tumor growth and metastasis, high-throughput functional assays, novel imaging techniques, single-cell vs. population-level cell behavior, and the role of the peritumoral microenvironment in cancer immunotherapy.
Colin D. Paul  
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*Department of Chemical and Biomolecular Engineering • Institute for NanoBioTechnology*  
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Website: colindpaul.weebly.com

**EDUCATION**

**Ph.D. in Chemical and Biomolecular Engineering**, Johns Hopkins University (Baltimore, MD)  
August 2010 – September 2015  
GPA: 3.96  
*Trainee, Nano-Bio Graduate Training Program, Johns Hopkins University Institute for NanoBioTechnology*  
*Ph.D. thesis: Physical mechanisms and high throughput imaging of tumor cell migration in confining microenvironments*  
*Advisor: Dr. Konstantinos Konstantopoulos*

**B.S. Chemical Engineering, B.S. Physics, Mathematics Minor**, University of Arkansas (Fayetteville, AR)  
May 2010  
GPA: 4.00  
*Summa Cum Laude, Tau Beta Pi, Phi Beta Kappa*  
*Undergraduate honors thesis: Pervaporative recovery of butanol from a simulated fermentation broth*  
*Advisor: Dr. Jamie Hestekin*

**RESEARCH EXPERIENCE**

**Konstantopoulos Lab, Johns Hopkins University**

- **Probing cellular traction forces in confined microenvironments**
  - Integrated micropost traction force and confined migration assays to elucidate biophysics of cell migration in confined microenvironments  
  - Created custom 3D-printed parts for microfluidic device alignment
- **Lens-free imaging for cell motility assays**
  - Led multinational, collaborative project between Johns Hopkins and imec (Leuven, Belgium) to establish lens-free imaging as an affordable and easy-to-use alternative to phase contrast imaging  
  - Optimized and benchmarked cell motility assays for lens-free imaging
- **Dimensionality and contact guidance in tumor cell migration**
  - Designed and fabricated (CAD, hard- and soft-lithography) microfluidic device to examine cell contact guidance, decision making, and metastatic propensity of cancer cell lines and tumor xenografts  
  - Isolated migratory cells and demonstrated their increased likelihood to form metastases in murine models  
  - Discovered crucial role of actomyosin contractility and RhoGTPase function in cellular contact guidance through use of pharmacological inhibition and siRNA-mediated gene knockdown  
  - Extensively quantified microenvironmental influences on cell morphodynamics  
Hestekin Lab, University of Arkansas (January 2008-December 2009)

- Dehydration of water-alcohol systems by pervaporation
  - Assembled bench-scale unit to study pervaporation as a means to dehydrate and purify butanol
  - Classified mixed matrix (PDMS/zeolite) membrane structure and performance using scanning electron microscopy and flux experiments

**TECHNICAL SKILLS**

**Cell culture and molecular biology:**
- Mammalian cell culture, transient and stable transfection, plasmid production, Western blotting, flow cytometry, DELFIA® binding assays

**Microscopy:**
- Time lapse imaging, epifluorescence and confocal microscopy, scanning electron microscopy, lens-free imaging

**Microtechnology:**
- Microfluidic device design, 3D printing, photolithography, soft lithography, microcontact printing

**Technical software:**
- ImageJ, MATLAB, COMSOL, Nikon NIS-Elements, Zeiss ZEN, GraphPad, AutoCAD, Autodesk Inventor

**AWARDS AND HONORS**

**Predoctoral Fellowships**
- Johns Hopkins University Schwarz Fellowship (August 2010-September 2015)
- NSF Graduate Research Fellowship (May 2011-April 2014)
- Integrative Graduate Education and Research Training (IGERT) in Physical and Biomolecular Foundations for Designing Nanoprobes Program, Johns Hopkins University (January 2011-May 2012)

**Predoctoral Awards and Honors**
- Johns Hopkins Chemical and Biomolecular Engineering Graduate Student Award (2015)
- Biomedical Engineering Society Graduate Design and Research Award (2013)
  - *A Microfluidic Device to Measure Traction Forces During Confined Migration*; received conference registration, $500 in travel support, and recognition at awards ceremony
  - Received travel and board support for research at IMEC (Leuven, Belgium); designed on-going collaborative research project
- Journal of Cell Biology paper selected to Faculty1000Prime (2013)
- Institute for NanoBioTechnology Nano-Bio Graduate Training Program (January 2011-present)
Selected Undergraduate Awards and Honors

- Senior Scholar, University of Arkansas; recognition for 4.0 grade-point average (2010)
- Outstanding Senior, Department of Chemical Engineering, University of Arkansas; award given to recognize outstanding academic performance, research, and service to the department (2010)
- 1st place, WERC Environmental Design Competition Task 4 (2010)
  o Improved Pretreatment for Shipboard RO Systems; led research arm of design team
- Arkansas Department of Education State Undergraduate Research Fellowship (January-May 2009)
  o Dehydration of Water-Alcohol Systems by Pervaporation; received $1250 stipend and $1000 in research cost support
- University of Arkansas Honors College Research Grant (August-December 2008)
  o Mixed Matrix Membranes for Recovery and Dehydration of Ethanol and Butanol; received $1250 stipend and $1000 in research cost support
- 1st place, American Institute of Chemical Engineers Mid-America Regional Conference Undergraduate Student Paper Competition (2009)
  o Pervaporative Extraction of Butanol from Fermentation Broth; led to invitation for platform presentation at 2009 American Institute of Chemical Engineers Annual Student Conference
- NSF Research Experience for Undergraduates, Iowa State University BioMaP REU (June-August 2009)
  o Multiblock Copolymers for Cartilage Engineering and Drug Delivery
- Honorable mention, Goldwater Scholarship Competition (2009)
- American Institute of Chemical Engineers Foundation Sophomore Academic Excellence Award, University of Arkansas; one recipient chosen per institution per year (2009)
- Arkansas Department of Education State Undergraduate Research Fellowship (January-May 2008)
  o Dehydration of Water-Alcohol Systems by Pervaporation; received $1250 stipend and $1000 in research cost support
- Honors College Fellow, University of Arkansas; received full tuition and living expenses (2006-2010)
- National Merit Scholarship, University of Arkansas (2006)

PUBLICATIONS

In Preparation


PRESENTATIONS
* indicates presenting author


### PATENTS


### TEACHING AND MENTORING EXPERIENCE

- **Mentor**, Konstantopoulos Lab, Department of Chemical and Biomolecular Engineering, Johns Hopkins University
  
  *This role includes training in project design, experimental techniques, and data analysis and presentation.*
  
  - Spring 2015: (2) Chemical and Biomolecular Engineering undergraduate students
  - Fall 2014: (3) Chemical and Biomolecular Engineering undergraduate students
  - Summer 2014: (1) Chemical and Biomolecular Engineering undergraduate student
  - Spring 2014: (2) Chemical and Biomolecular Engineering undergraduate students
  - Fall 2014: (2) Chemical and Biomolecular Engineering undergraduate students
  - Spring 2013: (1) Chemical and Biomolecular Engineering undergraduate student
  - Fall 2013: (1) Chemical and Biomolecular Engineering undergraduate student
  - Summer 2013: (1) Chemical and Biomolecular Engineering undergraduate student
  - Spring 2013: (2) Chemical and Biomolecular Engineering undergraduate students
  - Fall 2012: (2) Chemical and Biomolecular Engineering undergraduate students

- **Participant**, Whiting School of Engineering Center for Leadership Engineering, Johns Hopkins University
  
  *Completed professional development courses in:*
  
  - The people side of work: management, conflict resolution and negotiation (Fall 2014)
  - Proposal writing (Spring 2014)
  - Business plan development (Fall 2013)
  - Resume and job application preparation (Fall 2013)

- **Teaching Assistant**, Institute for NanoBioTechnology, Johns Hopkins University
  
  *NanoBioTechnology Lab (Spring 2013)*
  
  - Led hands-on lab courses focusing on microfabrication and microcontact printing for cell motility studies
• **Teaching Assistant**, Department of Chemical and Biomolecular Engineering, Johns Hopkins University
  
  *Transport Phenomena I, with Dr. Konstantinos Konstantopoulos (Spring 2012 and Spring 2013)*
  
  - Gave course lectures, led weekly help sessions, and graded exams

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**PROFESSIONAL SERVICE**

- **Writer**, Johns Hopkins University Nano-Bio Magazine
  
  *Johns Hopkins University (Spring 2013)*
  
  - Wrote article detailing STEM outreach activities sponsored by the Johns Hopkins Institute for NanoBioTechnology

- **Co-chair**, Institute for NanoBioTechnology Fall Symposium
  
  *Johns Hopkins University (October 2012)*
  
  - Developed program of graduate student, postdoctoral fellow, and professor presentations
  
  - Led spotlight session on National Cancer Institute (NCI) Physical Sciences-Oncology Center and Centers of Cancer Nanotechnology Excellence programs with NCI representative

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**LEADERSHIP EXPERIENCE**

- **Volunteer**, Incentive Mentoring Program (IMP)
  
  *Johns Hopkins University (2010-present)*
  
  - Mentored and tutored underperforming Baltimore City High School students
  
  - Worked as IMP “Grandparent,” leading three teams of 3-6 volunteers per team to provide academic and social support to IMP students; role included monthly leadership training (2011-2013)
  
  - Organized and ran Science Day (co-sponsored with JHU Institute for NanoBioTechnology) to introduce IMP students to science careers (2013)
  
  - Inducted to IMP Hall of Fame in 2014 for outstanding contributions to the program

- **Sports Chair**, Graduate Student Liaison Committee, Department of Chemical and Biomolecular Engineering
  
  *Johns Hopkins University (2011-2013)*
  
  - Organized departmental intramural sports teams
  
  - Attended Committee board meetings to serve as graduate student liaison with faculty and plan social and recruitment events

- **Volunteer**, STEM Science Outreach Program
  
  *Johns Hopkins University (2010)*
  
  - Prepared science demonstrations for middle school students at after-school recreation centers to encourage careers in the sciences

- **Project Leader**, Make a Difference Day
  
  *University of Arkansas (2008)*
  
  - Led a team of five volunteers to refurbish a garden at a local community center
• **President**, American Institute of Chemical Engineers Student Chapter  
  *University of Arkansas (2007-2008)*  
  o Organized and ran AIChE chapter meetings

• **Vice President and Treasurer**, Undergraduate Math Club  
  *University of Arkansas (2006-2009)*  
  o Drafted budgets and processed purchase orders, organized lectures, and wrote constitution and officer guide

• **Member** of the following professional societies:  
  o American Institute of Chemical Engineers (AIChE)  
  o Biomedical Engineering Society (BMES)

**PROFESSIONAL EXPERIENCE**

• **Process Engineering Intern**, CP Kelco (Okmulgee, OK)  
  *May 2009-August 2009*  
  o Created process flow diagrams of biogum production process and plant utilities for Six Sigma analysis of plant efficiency;  
  o Quantified biogum losses in effluent for plant mass balance

**HONORS SOCIETY INDUCTIONS**

• Phi Beta Kappa, Academic Honors Society (University of Arkansas, 2010)  
• Sigma Pi Sigma, Physics Honors Society (University of Arkansas, 2010)  
• Tau Beta Pi, Engineering Honors Society (University of Arkansas, 2007)  
• Sigma Pi Sigma, Physics Honors Society (University of Arkansas, 2010)  
• Phi Kappa Phi, Academic Honors Society (University of Arkansas, 2007)