SINGLE-CELL APPROACHES TO ASSESS HEMATOPOIETIC STEM CELL RESPONSE TO MATRIX CUES

BY

JI SUN CHOI

DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemical Engineering in the Graduate College of the University of Illinois at Urbana-Champaign, 2014

Urbana, Illinois

Doctoral Committee:

Assistant Professor Brendan A. C. Harley, Chair
Professor Brian Cunningham
Professor Deborah Leckband
Associate Professor Mary Kraft
ABSTRACT

Hematopoietic stem cells (HSC) are adult stem cells responsible for life-long hematopoiesis, the production of the full complement of blood and immune cells for hematological and immune systems. HSCs reside in the bone marrow within specialized microenvironments known as niches, comprised of various cellular and extracellular components including niche cells, extracellular matrix (ECM), and immobilized or soluble signaling molecules that provide extrinsic cues to regulate HSC fate decisions: quiescence, self-renewal, differentiation, mobilization, migration, retention, and apoptosis. Variations in structure and composition across the marrow niche microenvironments are believed to present combinations of signals that selectively regulate multiple stages of hematopoiesis. As predicted by such a hypothesis, HSCs exhibit dynamic behavior (e.g., mobilization, homing, migration) and localize non-uniformly within the marrow, with more primitive HSCs frequently localizing near the endosteum while less primitive, cycling progenitors associated with vascular sinusoids. The dynamic interplay between HSCs and a range of subregions within the bone marrow suggest that microenvironmental signals may be key regulators of HSC fate decisions. However, teasing apart the effect of niche-inspired biophysical cues on HSC fate decision processes have been challenging due to the rarity of HSCs within the marrow, our lack of technical ability to expand or differentiate HSCs freely in vitro, and a lack of available single cell metrics to continuously analyze the functional output of HSCs in situ. Our goal therefore is to build a multiplex in vitro culture platform that provides defined matrix cues while enabling label-free analysis of HSCs in situ. We first show that matrix cues do in fact directly impact HSC biophysical properties (Chapter 2) and fate decisions (Chapter 3) and identify a set of matrix cues that favor HSC self-renewal over lineage specification (Chapter 3). We then evaluate single cell analysis techniques
(e.g., Raman Spectroscopy) as potential functional metrics for HSCs (Chapter 4). In particular, we assess the feasibility of using adhesive signatures of individual HSCs (e.g., Photonic crystal enhanced microscopy) as a new metric for monitoring their functional output (Chapter 5). Finally, we review our progress and discuss ongoing work and future opportunities (Conclusions).
ACKNOWLEDGEMENTS

Thank you so much to everyone whose paths have crossed mine during my 6 years of graduate school life at the University of Illinois at Urbana-Champaign. My experiences and interactions with you all not only taught me how to be an independent researcher but shaped me into the person that I am today.

First and foremost, I would like to thank my advisor Prof. Brendan Harley for amazing guidance, support, and encouragement throughout my PhD. I would like to also thank the current and past members of the Harley Lab for meaningful discussions, playful banter, and fun times in and outside of lab: Bhushan Mahadik, Steven Caliari, Sara Pedron, Rebecca Hortensius, Dan Weisgerber, Laura Mozdzen, Bill Grier, Jackie Pence, Emily Gonnerman. (You guys are the best!) I should also acknowledge my wonderful undergraduates Tyler Leonard, Roxanne de Leon, and Lucas Tan who offered tremendous help with rheological measurements and hydrogel fabrication.

I am grateful to my collaborators across campus for the lovely conversations and collaborations that greatly enhanced my technical insight. I want to particularly thank Jessica Frisz, Robbie Wilson, and Yelena Ilin from Kraft Lab, Erich Lidstone, Yue Zhuo, Kenny Long, Weili Chen, and Hojeong Ryu from Cunningham Lab, and Xuefeng Wang from Ha Lab for invaluable learning opportunities. I thoroughly enjoyed working with you all.

I would like to thank my committee members, Prof. Brian Cunningham, Prof. Mary Kraft, and Prof. Deborah Leckband for continued support and helpful feedback in completing my PhD research and thesis. I also want to thank Barbara Pilas, Ben Montez, Bill Hanafin at the Flow Lab for assistance with flow cytometry, Scott Maclaren and Jenny Amos for training me on AFM, and Shiv Mayandi for assisting me with imaging analyses.
This past year, I was blessed with an opportunity to work as a commercialization analyst intern at the OTM office. I would like to thank the OTM director Leslie Millar-Nicholson, TMs (Jen Rice, Jeff Wallace, Lisa Dhar, Brad Edwards, Nate Hoffman, Steve Wille, Svetlana Sowers), staff members, and my fellow interns (Meredith Walker, Courtney Cox, Jason Hale, Gabe Ejezie, Mark Scott, Bob Bunton, Ryan Richardson, Diego Martin De La Fuente, Wanjing Li, Paul VanderVelde) for the wonderful times at OTM.

Last but not least, I want to thank my family and friends. I love you, Mom and Dad and my little brother Hong Ik! Thank you for always believing in me. Thank you to my friends at the U of I who have supported me throughout. I’d like to especially thank So Youn Kim, Rayna Kim, Minseok Jang, and Leslie Hwang for always being there for me. There are so many other great people I have gotten to know at the U of I and I apologize for not being able to name everyone here but you know who you are!

Funding sources that supported my PhD research include ChBE department fund, Illini 4000, IGB fund, American Cancer Society of Illinois #160673 and #189782, and National Science Foundation, CBET 1254738 (CAREER).
TABLE OF CONTENTS

LIST OF SYMBOLS AND ABBREVIATIONS ........................................................................................................ IX

CHAPTER 1: INTRODUCTION .......................................................................................................................... 1

1.1. HEMATOPOIETIC STEM CELLS (HSC) ........................................................................................................ 1
1.2. HSC Niches in the Bone Marrow .................................................................................................................. 3
  1.2.1. Endosteal Niche ........................................................................................................................................ 5
  1.2.2. Perivascular Niche .................................................................................................................................... 6
  1.2.3. Other Putative Marrow Niches ................................................................................................................ 8
1.3. CURRENT CHALLENGES IN THE FIELD ..................................................................................................... 9
1.4. EXTRINSIC CONTROL OF HSC FATE DECISIONS IN VITRO .................................................................... 10
  1.4.1. Cytokines .................................................................................................................................................. 12
  1.4.2. Co-Culture with Niche Cells .................................................................................................................... 13
  1.4.3. Matrix Cues on HSC Fate Decisions ......................................................................................................... 13
1.5. IN VITRO CULTURE PLATFORMS FOR HSCS .......................................................................................... 15
1.6. FIGURES ..................................................................................................................................................... 19
1.7. TABLES ..................................................................................................................................................... 24

CHAPTER 2: IMPACT OF MATRIX ELASTICITY, CONSTRUCT DIMENSIONALITY, AND LIGAND DENSITY ON THE VIABILITY AND BIOPHYSICAL PROPERTIES OF HEMATOPOIETIC STEM AND PROGENITOR CELLS ............................................................................ 26

2.1. ABSTRACT .................................................................................................................................................... 26
2.2. INTRODUCTION .......................................................................................................................................... 27
2.3. MATERIALS AND METHODS ........................................................................................................................ 29
  2.3.1. Preparation and collagen functionalization of 2D polyacrylamide substrates ............................................. 30
  2.3.2. 3D collagen hydrogel preparation ............................................................................................................. 31
  2.3.3. Mechanical characterization ....................................................................................................................... 32
  2.3.4. Hematopoietic stem and progenitor cell isolation and culture ................................................................. 33
  2.3.5. Culture of 32D cells .................................................................................................................................... 34
  2.3.6. Viability assays .......................................................................................................................................... 35
  2.3.7. Immunofluorescence staining and confocal imaging ................................................................................. 35
  2.3.8. Image analysis of cell size, shape, and cytoskeletal organization ............................................................ 36
  2.3.9. Statistical analysis .................................................................................................................................... 37
2.4. RESULTS ..................................................................................................................................................... 37
  2.4.1. Mechanical characterization of collagen hydrogels .................................................................................... 37
  2.4.2. Mechanical characterization of polyacrylamide (PA) substrates ............................................................... 38
  2.4.3. HSPC isolation ......................................................................................................................................... 38
  2.4.4. Effect of substrate dimensionality on HSPC biophysical properties ......................................................... 39
  2.4.5. Effect of substrate stiffness on HSPC viability and morphology ............................................................. 40
  2.4.6. Effect of substrate collagen density on HSPC biophysical properties .................................................... 41
  2.4.7. Effect of substrate stiffness on the biophysical properties of 32D cells .................................................... 41
2.5. DISCUSSION ............................................................................................................................................... 42
2.6. CONCLUSIONS ........................................................................................................................................... 48
2.7. FIGURES ....................................................................................................................................................... 50
2.8. TABLES ....................................................................................................................................................... 59
CHAPTER 3: MATRIX CUES (STIFFNESS, LIGAND PRESENTATION) RAPIDLY IMPACT FATE DECISIONS OF HEMATOPOIETIC STEM AND PROGENITOR CELLS .......................................................................................................................... 60

3.1. ABSTRACT ........................................................................................................... 60
3.2. INTRODUCTION ................................................................................................. 60
3.3. MATERIALS AND METHODS .............................................................................. 63
  3.3.1. Substrate preparation ....................................................................................... 63
  3.3.2. HSC isolation .................................................................................................. 65
  3.3.3. Cell culture ...................................................................................................... 65
  3.3.4. Flow cytometric analysis ................................................................................. 66
  3.3.5. Colony-forming unit (CFU) assay .................................................................... 66
  3.3.6. Statistical analysis .......................................................................................... 67
3.4. RESULTS ............................................................................................................. 67
  3.4.1. Biophysical changes in HSCs occur within 24h of culture and are linked to matrix environment .................................................................................................................. 68
  3.4.2. Functional changes in HSCs after initial matrix contact .................................. 68
  3.4.3. Functional changes in HSCs as a function of matrix stiffness and ligand presentation .......................................................... 70
  3.4.4. Actomyosin contractility impacts early HSC fate.............................................. 71
  3.4.5. Integrin activation on the regulation of early HSC fate .................................... 73
3.5. DISCUSSION ....................................................................................................... 74
3.6. CONCLUSIONS ................................................................................................. 77
3.7. FIGURES ............................................................................................................. 78
3.8. TABLES ............................................................................................................... 93

CHAPTER 4: SINGLE CELL ANALYSIS METHODS FOR QUANTIFYING FUNCTIONAL OUTPUT OF HEMATOPOIETIC STEM CELLS ................................................................. 95

4.1. GENERAL CHAPTER OVERVIEW ...................................................................... 95
4.2. ATOMIC FORCE MICROSCOPY ....................................................................... 96
  4.2.1. Abstract ......................................................................................................... 96
  4.2.2. Introduction .................................................................................................... 97
  4.2.3. Materials and Methods .................................................................................. 99
  4.2.4. Results ............................................................................................................ 103
  4.2.5. Discussion ..................................................................................................... 105
  4.2.6. Conclusions .................................................................................................. 106
4.3. TIME-OF-FLIGHT SECONDARY ION MASS SPECTROSCOPY (TOF-SIMS) ............. 107
  4.3.1. Abstract ......................................................................................................... 107
  4.3.2. Introduction .................................................................................................... 108
  4.3.3. Materials and Methods .................................................................................. 110
  4.3.4. Results ............................................................................................................ 111
  4.3.5. Discussion and Conclusions .......................................................................... 117
4.4. RAMAN SPECTROSCOPY .................................................................................. 119
  4.4.1. Abstract ......................................................................................................... 119
  4.4.2. Materials and Methods .................................................................................. 120
  4.4.3. Preliminary Results ......................................................................................... 122
  4.4.4. Ongoing Work ................................................................................................ 122
4.5. FIGURES ............................................................................................................. 124
4.6. TABLES ............................................................................................................... 139
CHAPTER 5: ADHESIVE SIGNATURES OF HEMATOPOIETIC STEM AND PROGENITOR CELLS AS A NEW SINGLE CELL METRIC ........................................ 146

5.1. GENERAL CHAPTER OVERVIEW ........................................................................................................ 146
5.2. PHOTONIC CRYSTAL ENHANCED MICROSCOPY (PCEM) .................................................. 147
  5.2.1. Abstract .................................................................................................................................. 148
  5.2.2. Introduction .......................................................................................................................... 148
  5.2.3. Materials and Methods ........................................................................................................ 150
  5.2.4. Results .................................................................................................................................. 152
  5.2.5. Discussion and Conclusions ................................................................................................. 160
5.3. TENSION GAUGE TETHER (TGT) SYSTEM AS ADHESIVE FORCE REPORTERS ............ 162
  5.3.1. Abstract .................................................................................................................................. 162
  5.3.2. Materials and Methods ........................................................................................................ 162
  5.3.3. Preliminary Results and Ongoing Work ............................................................................... 164
5.3. FIGURES ...................................................................................................................................... 166

CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS ................................................. 174

6.1. CONCLUSIONS ............................................................................................................................ 174
6.2. FUTURE DIRECTIONS ................................................................................................................ 176

APPENDIX A: GEL FABRICATION AND CHARACTERIZATION PROTOCOLS .... 177

A.1. COLLAGEN HYDROGEL FABRICATION PROTOCOL ................................................................. 177
A.2. COLLAGEN HYDROGEL CELL ENCAPSULATION PROTOCOL ................................................. 178
A.3. SEM IMAGING OF COLLAGEN HYDROGEL PROTOCOL .......................................................... 179
A.4. COLLAGEN HYDROGEL MECHANICAL CHARACTERIZATION PROTOCOL .................... 180
A.5. PA GEL FABRICATION PROTOCOL .......................................................................................... 181
A.6. PA GEL SURFACE FUNCTIONALIZATION PROTOCOL ............................................................... 183
A.7. AFM FORCE MEASUREMENT PROTOCOL .............................................................................. 184

APPENDIX B: CELL CULTURE AND ANALYSIS PROTOCOLS ........................................ 188

B.1. HSC ISOLATION PROTOCOL ....................................................................................................... 188
B.2. FIXED CELL STAINING PROTOCOL .......................................................................................... 191
B.3. IMAGEJ: CELL SPREAD AREA CALCULATION PROTOCOL .................................................. 192

REFERENCES ...................................................................................................................................... 194
# LIST OF SYMBOLS AND ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Term; Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
</tr>
<tr>
<td>Ang-1</td>
<td>Angiopoietin-1</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>CAFC</td>
<td>Cobblestone area-forming cell assay</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony-forming unit assay</td>
</tr>
<tr>
<td>CLP</td>
<td>Common lymphoid progenitor</td>
</tr>
<tr>
<td>CMP</td>
<td>Common myeloid progenitor</td>
</tr>
<tr>
<td>Col</td>
<td>Collagen type I</td>
</tr>
<tr>
<td>CSI</td>
<td>Cell shape index</td>
</tr>
<tr>
<td>CSK</td>
<td>Cytoskeletal</td>
</tr>
<tr>
<td>CXCL12</td>
<td>Chemokine (C-X-C motif) ligand 12</td>
</tr>
<tr>
<td>DLL4</td>
<td>Delta-like ligand 4</td>
</tr>
<tr>
<td>E</td>
<td>Elastic modulus</td>
</tr>
<tr>
<td>EdU</td>
<td>5-ethynyl-2′-deoxyuridine</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FGF-4</td>
<td>Fibroblast growth factor-4</td>
</tr>
<tr>
<td>Flt3L</td>
<td>Fms-related tyrosine kinase 3-ligand</td>
</tr>
<tr>
<td>Fn</td>
<td>Fibronectin</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>GVHD</td>
<td>Graft-versus-host disease</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
</tr>
<tr>
<td>HSPC</td>
<td>Hematopoietic stem and progenitor cell</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>Lepr</td>
<td>Leptin receptor</td>
</tr>
<tr>
<td>Ln</td>
<td>Laminin</td>
</tr>
<tr>
<td>LSK</td>
<td>Lin−Sca-1&quot;c-Kit+</td>
</tr>
<tr>
<td>LTC-IC</td>
<td>Long-term culture-initiating cell assay</td>
</tr>
<tr>
<td>LT-HSC</td>
<td>Long-term hematopoietic stem cell</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MPP</td>
<td>Multipotent progenitor cell</td>
</tr>
<tr>
<td>NK cell</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>PA</td>
<td>Polycrylamide</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal component analysis</td>
</tr>
<tr>
<td>PCEM</td>
<td>Photonic crystal enhanced microscopy</td>
</tr>
<tr>
<td>Pml</td>
<td>Promyelocytic leukemia protein</td>
</tr>
<tr>
<td>PLS-DA</td>
<td>Partial least-squares discriminant analysis</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>PTHrP</td>
<td>PTH-related protein</td>
</tr>
<tr>
<td>PPR</td>
<td>PTH/PTHrP receptors</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Term; Definition</td>
</tr>
<tr>
<td>-------------</td>
<td>------------------</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem cell factor</td>
</tr>
<tr>
<td>ST-HSC</td>
<td>Short-term hematopoietic stem cell</td>
</tr>
<tr>
<td>TGT</td>
<td>Tension gauge tether</td>
</tr>
<tr>
<td>TOF-SIMS</td>
<td>Time-of-flight secondary ion mass spectroscopy</td>
</tr>
<tr>
<td>TPO</td>
<td>Thrombopoietin</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>VEGF receptor</td>
</tr>
</tbody>
</table>
CHAPTER 1: Introduction

1.1. Hematopoietic Stem Cells (HSC)

Hematopoietic stem cells (HSC) are adult stem cells with the capacity to self-renew indefinitely or differentiate to become lineage-restricted cells. HSCs are responsible for life-long hematopoiesis, the production of the full complement of blood and immune cells for hematological and immune systems (1-6). This rare population of adult stem cells (<1:50,000 marrow cells) resides primarily in the bone marrow to dynamically supply differentiated progeny with high turnover rates, from neutrophils whose half-life is only 8 hours to erythrocytes whose half-life is 120 days (3). This is enabled by the tight regulation of HSC fate that results in a hierarchy of stem and progenitor cells with distinct differentiation and engraftment potential, from long-term HSCs (LT-HSC) with indefinite self-renewal capacity to short-term HSCs (ST-HSC) and multipotent progenitor cells (MPP) with limited self-renewal ability and terminally-differentiated cells (1). The delicate balance between stem cells and lineage-restricted progenitors permits the constant generation of blood and immune cells for the body, including macrophages, granulocytes, erythrocytes, megakaryocytes, platelets, T cells, B cells, and NK cells (Fig. 1.1).

HSCs reside in specialized microenvironments known as niches, comprised of various cellular and extracellular components including niche cells, extracellular matrix (ECM), and immobilized or soluble signaling molecules (1-9). HSCs closely interact with a range of these niche constituents that provide extrinsic cues, which drives intrinsic changes in gene expressions and/or epigenetic regulations to impact critical HSC fate decisions such as quiescence, self-renewal, differentiation, mobilization, migration, retention, and apoptosis (7). Extrinsic and intrinsic regulatory cues are believed to provide instructive signals to mediate HSC fate decision
processes required to maintain a constant pool of quiescent stem cells as well as differentiated progeny.

HSCs have been utilized in bone marrow transplants since the 1970s to treat bone marrow malignancies including leukemia, lymphoma, and myeloproliferative disorders (10). Because HSCs have the capacity to give rise to all blood and immune cells, donor bone marrow can successfully reconstitute the patient bone marrow upon transplantation following myeloablative chemotherapy or radiation therapies (10-13). Yet, difficulties in identifying a matching donor or securing an otherwise appropriate source of the marrow (e.g., umbilical cord blood, peripheral blood, donor marrow), possible immune response (e.g., graft-versus-host disease: GVHD), other complications (e.g., interstitial pneumonitis and other lung problems), and high costs are often the limiting factors of a typical allogeneic bone marrow (10). Although clinical outcome varies with disease type, patient age, and associated risk factors, one-year survival rate after receiving allogeneic bone marrow transplant currently remains at around 65% in children with acute leukemia (14). During this period of time, successful transplants are characterized by donor HSCs able to stably engraft within recipient marrow cavities, though transfusions of blood products, particularly platelets and erythrocytes, are required before, during, and after bone marrow transplantation (15). Once patients survive for 2 years post-transplant, the survival rate rises to ~85%, with recurrent disease and chronic GVHD being two primary causes of mortality (16). Clinically, evidence suggests that the number of HSCs (CD34+ cells) present in the transplanted marrow is linked to patient survival and time to engraftment (17-19). Being able to engineer HSC fate in vitro to expand a pool of HSCs prior to infusion or obtain differentiated marrow populations such as platelets would thus have the potential to make bone marrow transplantation more accessible, increase engraftment efficiency, reduce associated risks, and
enable more autologous transplants. Better understanding of the HSC fate decision processes may also lend insight regarding pathological mechanisms associated with bone marrow malignancies, such as bone marrow failure, that are often the result of defective HSC fate regulating mechanisms.

1.2. HSC Niches in the Bone Marrow

HSCs reside primarily in the bone marrow cavities of the trabecular bone (1). Enclosed by the dense compact bone, the highly vascularized trabecular bone and the soft marrow serve as the principal sites of hematopoiesis (Fig. 1.2) (20). In rodents, which are most frequently used to study hematopoiesis and HSCs, long bones such as femurs and tibias serve as the predominant sites for life-long hematopoiesis (2). In contrast, these sites cease to mediate hematopoiesis in adult humans as adipocytes increasingly occupy the marrow space (yellow marrow), with the exception of the proximal regions that retain high numbers of hematopoietic cells (red marrow) (21). Instead, the bones of the axial skeleton such as the cranium, sternum, ribs, vertebrae, and the iliac crest in the pelvis serve as the major sites of hematopoiesis in humans (2).

Previous studies have revealed significant structural and mechanical heterogeneity across the bone marrow (Fig. 1.2) (20, 22-26). Notably, confinement within the marrow exposes cells to a range of stiffness, from the stiffer region near the bone surface composing of osteoids and collagenous bone ($E_{\text{collagenous bone}} = 40$-$50$ kPa) to the marrow comprising of adipocytes and various other cell types ($E_{\text{cell membrane}} \leq 3$ kPa) (23, 24, 27). Likewise, ECM protein presentation and density vary significantly across the marrow, where higher levels of fibronectin are found near the endosteum while higher expressions of laminin are associated with the marrow vasculature (Table 1.1) (26). Additionally, the local composition such as biomolecular cues (e.g.,
hypoxia, chemokine (C-X-C motif) ligand 12 (CXCL12), cytokines such as stem cell factor (SCF), Ca\(^{2+}\) and local cell populations (e.g., osteoblasts, mesenchymal stem cells (MSC), vascular endothelial cells, differentiated hematopoietic progeny) varies considerably across the marrow (1-9). The bone marrow is also subject to varying degrees of biophysical forces, including hydrostatic pressure and fluid shear stress (20, 24), with HSCs also experiencing significant fluid shear stresses during regular mobilization to and circulation within the peripheral blood (28).

Variations in structure and composition across the bone marrow present distinct combinations of extrinsic signals that may selectively contribute to hematopoiesis by favoring certain HSC fate decisions over others. As predicted by such a hypothesis, HSCs exhibit dynamic behavior within the marrow, with quiescent versus activated HSCs homing, mobilizing to/from the marrow into the peripheral blood (4, 8, 29, 30). Also, hematopoietic progenitor cells localize non-uniformly within the marrow at discrete stages of lineage specification, with more primitive hematopoietic stem and progenitors frequently localizing near the endosteum while less primitive, cycling progenitors associate with sinusoids, away from the endosteum (25, 31-33). In addition, evidence suggests that bone disease, aging or disuse alters the compositional properties of the marrow (8, 20). The dynamic interplay between HSCs and the diverse marrow microenvironments suggest that microenvironmental signals may play a key role in regulating HSC fate decisions such as *quiescence, self-renewal, and myeloid vs. lymphoid lineage specification* (24, 34). These observations further suggest that hematopoietic homeostasis requires a balance between a small number of *quiescent niches* and more prevalent *active niches* to regulate HSC numbers and their functions (2).
Over the past two decades, the subject of identifying the HSC niche has inspired a multitude of investigations. While the identity and composition of discrete niches remains unclear, putative HSCs have been identified near the bone endosteum (*endosteal* niche), in the central marrow cavity (*central medullary* niche) or the sinusoidal endothelium (*perivascular* niche) (1, 2, 4, 8, 25, 31) (**Fig. 1.2**).

### 1.2.1. Endosteal Niche

Data suggests that the *endosteal* niche houses quiescent LT-HSCs and may mediate long-term maintenance of the HSC pool (1, 2, 4, 8). A strong piece of evidence for the existence of the *endosteal* niche was established from the observations that premature osteoblasts, which highly populate the endosteum, can maintain HSCs and enhance the HSC pool (35-37). For example, human osteoblastic cells could support long-term culture-initiating cells (LT-CIC) and progenitors in *ex vivo* cultures (35). In 2003, Calvi *et al.* showed constitutive activation of parathyroid hormone (PTH)/PTH-related protein (PTHrP) receptors (PPRs) in osteoblasts of transgenic mice leads to an increase in the HSC pool size via jagged 1-Notch signaling (36). Similarly, another report in 2003 by Zhang *et al.* showed that conditional inactivation of bone morphogenetic protein (BMP) receptor 1a expands osteoblasts and increases HSC numbers (37). Interestingly, different subsets or stages of osteoblasts were found to have a differing role in HSC regulation. While constitutive activation of PPRs in mature osteoblasts (*i.e.*, osteocytes) had no impact on HSC maintenance or function (38), premature osteoblastic cells such as nestin\(^+\) MSCs (39) or Runx2\(^{hi}\) osbeoblasts (40) were shown to enrich HSCs by expressing high levels of angiopoietin-1 (ang-1), SCF, CXCL12, and other cytokines known to support HSC quiescence (41) and self-renewal (4, 39, 42).
The concept of a discrete *endosteal* niche was further supported by observed preferential homing and engraftment of primitive hematopoietic stem and progenitors to sites adjacent to the endosteum (25, 31, 32, 42, 43). In 2006, *Adams et al.* showed that HSCs depleted of calcium sensing capacity (calcium-sensing receptors (CaR<sup>−</sup>) HSCs) in antenatal mice fail to successfully lodge to the endosteum, a site rich in calcium ions (Ca<sup>2+</sup>) deposited by osteoblasts, and instead are found in circulation or in the spleen (43). More recently, improved imaging techniques allowed the direct visualization of hematopoietic stem and progenitor cells in relation to the endosteum (25, 31). In 2009, *Xie et al.* observed that GFP<sup>+</sup> HSCs preferentially localize to the endosteum in irradiated mice recipients via *ex vivo* imaging of the isolated femur (31). Similarly, in 2013, *Nombela-Arrieta et al.* demonstrated using an image cytometry approach able to map the distributions of >10<sup>5</sup> cells within the marrow that c-Kit<sup>+</sup> hematopoietic progenitor cells associate with the endosteal region (25).

### 1.2.2. Perivascular Niche

Found more deeply within the marrow, the sinusoidal endothelium (*perivascular* niche) has been implicated in housing more active, self-renewing ST-HSCs (1, 2, 4, 8). The possibility of the *perivascular* niche was first proposed based on 2005 studies (*Sipkins et al.*; *Kiel et al.*) that showed co-localization of hematopoietic stem and progenitor cells with the marrow vasculature (44, 45). Since then, imaging analyses have confirmed that hematopoietic stem and progenitor cells are in close proximity to or in contact with the marrow vessels, even when they are in the endosteal region (25, 31, 46). This is not surprising considering the dense vascularization within the bone marrow, as well as established HSC mobilization and homing processes. Structurally, the artery first enters the cortical bone to split into ascending and
descending branches that run axially along the central marrow cavity, then radially towards the endosteal region while giving rise to a network of smaller arterioles (Sca-1\(^+\)) and capillaries that anastomose with wider, irregularly-shaped venous sinusoids (Sca-1\(^{lb}\); diameter ~ 50-100 \(\mu m\)) (Fig. 1.3) (9, 22, 25, 46, 47). These venous sinusoids eventually drain via the central longitudinal vein that runs parallel to the artery in the marrow cavity, hence creating a circular flow of blood supply within the bone marrow (22). Due to the anatomical proximity of the bone to the vasculature, it is currently unclear if perivascular and endosteal niches exist as separate niche microenvironments or if they partially overlap.

Evidence suggests that vascular endothelial cells are critical for HSC maintenance and function (48-50). Bone marrow-derived endothelial cells could expand HSCs and support LT-HSCs in \textit{in vitro} cultures (48, 49) while non-hematopoietic endothelial cells showed varying degrees of HSC-supporting capacity (51, 52). Also, as Ding \textit{et al.} reported, conditional deletion of SCF from endothelial cells or leptin receptor (Lepr)-expressing perivascular stromal cells – but not from hematopoietic cells, osteoblasts, or nestin\(^+\) cells – decreased the frequency and impaired the long-term repopulating ability of HSCs (50). Nonetheless, \textit{perivascular} niche alone seems to be insufficient in supporting hematopoiesis because conditional ablation of osteoblastic cells (\textit{i.e.}, endosteal niche) results in extramedullary hematopoiesis in the spleen and the liver (53). Perivascular region most likely contributes to regulating the differentiation activity of HSCs and progenitors as reports indicate that these cells are recruited to the perivascular region following bone marrow stress (29, 30). For example, Heissig \textit{et al.} reported that c-Kit\(^+\) hematopoietic stem and progenitor cells mobilize to the perivascular region following ablation of the bone marrow via matrix metalloproteinase 9 (MMP9)-mediated release of soluble SCF that attracts these cells and drive increased hematopoietic activity (29). Similarly, Avecella \textit{et al.}
found marrow vessels release cytokines (i.e., CXCL12, fibroblast growth factor-4 (FGF-4)) to attract hematopoietic stem and progenitor cells for megakaryocyte maturation and thrombopoiesis (30).

1.2.3. Other Putative Marrow Niches

Kunisaki et al. recently proposed that arterioles may also serve as a perivascular niche (arteriolar niche) that supports HSC quiescence (46). Using confocal imaging and computational modeling of the sternum, they found that 36.8% and 67.1% of CD150^+CD48^−CD41^−Lineage^− HSCs are located within 20-μm distance from arterioles (Sca-1^{hi}VEGFR2^+VEGFR3^−) and sinusoids, respectively, despite that arterioles and sinusoids only occupy 1.2% and 30% of the sternal bone marrow by volume. Interestingly, quiescent HSCs (44.3% of total HSCs, as indicated by 5-ethyl-2'-deoxyuridine (EdU) stain (EdU^+ HSCs)) visualized near arterioles were in close association with nestin^+GFP^{bright} perivascular cells, particularly NG2^+nestin^+GFP^{bright} cells. Administration of the known HSC-mobilizing agent granulocyte colony-stimulating factor (G-CSF) caused HSCs to migrate away from arterioles. Likewise, in mice deficient in promyelocytic leukemia protein (Pml^-/-) where HSC quiescence is compromised in a cell-autonomous fashion, HSCs were located significantly further away from arterioles, suggesting that arterioles are implicated in HSC quiescence.

Due to the anatomical proximity of the bone to the marrow vasculature, particularly arterioles to the endosteum, it is not clear if arteriolar niche highlights the importance of additional components within existing niche models (endosteal and perivascular niches) or present a new, mutually exclusive niche. Nonetheless, these subtle localization differences within the bone marrow support the contention that quiescent and active niches exist in close spatial
register across the marrow. Teasing apart the complex constellations of signals that define cell fate-determining niches remains a challenge due to the complexity of the marrow composition, rarity of HSCs within the marrow, and technical limitations regarding the ability to assess HSC fate via imaging techniques and functional metrics.

1.3. Current Challenges in the Field

While previous studies have revealed many aspects of hematopoiesis and potential regulatory aspects of HSC fate by the bone marrow niche microenvironments, the complete structure, composition, and role of the niche remains unclear due to technical limitations (3). First, it is challenging to identify bona fide HSCs. Previous reports recognized several characteristic cell surface antigens expressed on distinct subsets of putative HSCs and progenitors, allowing hematopoietic stem and progenitor sub-populations to be reproducibly isolated via fluorescence-activated cell sorting (FACS) (Fig. 1.4) (45, 54). Still, the exact sets of antigens expressed on subsets of HSCs according to their self-renewal capacity remain ambiguous, requiring a combination of a number of different cell surface markers for identification, especially to enrich in LT-HSCs. Such technical issues combined with the low frequency of HSCs within the marrow (Table 1.2) often results in putative HSCs described in the literature almost certainly being a heterogeneous population (55, 56) with only a very small number of them being true HSCs. Combined with limitations of in situ imaging (57), it is difficult to dynamically assess if alterations in microenvironmental conditions lead to real changes in HSC fate decisions or simply selectively expand certain lineage-restricted progenitor populations that already exist in the subpopulation under study.
Another major challenge in the field is moving past established functional assays. After decades of research in the field, repopulation assays still remains to be the gold standard for confirming the identity of a putative HSC (58). Here, successful reconstitution of the irradiated marrow upon introducing a donor HSC population validates its multi-lineage repopulating potential (58, 59). Other functional assays such as colony-forming unit (CFU) assay, long-term culture-initiating cell (LTC-IC) assay, and cobblestone area-forming cell (CAFC) assay are often used as a surrogate for the repopulation assay because they are more convenient and cost-effective (59). However, these still require long incubation time, often require large number of putative HSCs for testing, and fail to provide continuous, label-free analysis of putative HSCs in situ. Means to dynamically monitor single putative HSCs in situ in a label-free manner would therefore supply technical platforms to better understand the nuances of HSC fate decision processes.

1.4. Extrinsic Control of HSC Fate Decisions in vitro

The capacity to extrinsically control HSCs in in vitro settings has been an intense topic of research in the past decades (Fig. 1.4). The aim of extrinsic control of HSCs to engineer HSC fate decisions is two-fold, first is to expand our understanding of the underlying mechanisms of HSC rate regulation and second is to clinically utilize HSCs in stem-cell based therapies (12, 13). Although HSCs have been utilized for bone marrow transplantation since 1970s, securing large numbers of HSCs for efficient transplantation remains a challenge (10-13). Clinically, the number of HSCs (CD34+ human HSCs) present in transplanted marrow (e.g., umbilical cord blood) has been linked to successful engraftment and patient survival (17-19). Therefore, tools to enable selective ex vivo expansion of donor HSCs from a larger heterogeneous population of
donor marrow prior to infusion could significantly improve clinical outlook. Likewise, controlled differentiation of HSCs to myeloid (e.g., platelets, red blood cells (RBC), neutrophils) or lymphoid lineages (e.g., T cells, B cells, NK cells) would be beneficial for clinical applications such as blood transfusion (11). However, our lack of understanding how HSC fate decisions are extrinsically regulated early (*quiescence vs. self-renewal; self-renewal vs. lineage specification*) or later in the differentiation process (*maturation into terminal cell types*) currently limits our ability to effectively manipulate HSCs *in vitro*.

HSCs possess an incredible ability to self-renew, a potential exploited in traditional bone marrow transplantation (10-13). Although only a small number of HSCs are maintained at homeostasis, HSCs can self-renew up to 20-fold upon ablation of the marrow (60, 61). A single HSC can successfully reconstitute the entire marrow, where initially transplanted HSCs can self-renew by 8,400-fold after four successive serial transplantations (61). As such, the indefinite self-renewal capacity of HSCs has been demonstrated *in vivo* but its induction *in vitro* has remained a challenge. Research effort in the past decades has identified several cytokines, small molecules, stromal cells, and combinations thereof that could support HSCs *in vitro* but protocols for the reliable expansion of HSCs are yet to be discovered due to excessive, uncontrolled differentiation of the starting cell population that follows as early as the first division (12, 13). While genetic modifications of HSCs (e.g., *HOXB4*) to achieve amplification are also possible (62, 63), these are undesirable due to the introduction of retroviral vectors. Hence, optimal conditions that could reproducibly and indefinitely expand HSCs still need to be established.

Research effort to differentiate HSCs into specific lineages has also shown only limited success. While successful differentiation of HSCs into myeloid (64-67), lymphoid (68), or other
lineages (69-71) in presence of feeder cells or in in vivo settings has been reported, in vitro differentiation protocols without the use of a feeder cell layer have been almost absent.

1.4.1. Cytokines

Several cytokines have been shown to support ex vivo expansion of human and mouse HSCs although the optimal set of such cytokines that could reliably and continuously expand HSCs are yet to be defined (3, 12, 13). These include SCF, thrombopoietin (TPO), fms-related tyrosine kinase 3-ligand (Flt3L), interleukin-3 (IL-3), IL-6, IL-11, and G-CSF (3, 72-75), some of which have been previously shown to be involved in in vivo regulation of HSC quiescence (41) and self-renewal (50). HSCs cultured in combinations of these factors showed increased repopulating activity when transplanted into irradiated recipients although this ability was lost in longer-term cultures (> 9 days) (73). Interestingly, evidence suggests that immobilized forms of these cytokines (e.g., macrophage colony-stimulating factor, M-CSF (76); SCF (77, 78)) may provide better support for the expansion of HSCs than soluble forms by providing more potent signals.

In the context of directed lineage specification of HSCs, Miharada et al. recently reported that erythroblast enucleation could be achieved by culturing human CD34+ cells with only cytokines for four successive passages (65). Like with directed HSC self-renewal studies, the use of feeder cell co-culture remains the primary approach used in investigations attempting to induce directed differentiation of HSCs into lymphoid and myeloid lineages.
1.4.2. Co-Culture with Niche Cells

The use of stromal cells for the *ex vivo* expansion of HSCs is well-established (11, 12). Bone marrow-derived stromal cells and other stromal cell lines (76, 79), MSCs (80), osteoblasts (35), vascular endothelial cells (48, 49, 51), and other feeder cells (81) have been shown to support long-term HSC maintenance and expansion *in vitro*. Similarly, the use of feeder cells for the differentiation of HSCs into myeloid (*e.g.*, RBCs (64), megakaryocytic cells (66)), lymphoid (*e.g.*, NK cells (67), B cells (68), T cells (82)), and other lineages (*e.g.*, hepatocytes (71), smooth muscle cells (SMC) (70)) have been reported. Notably, Taqvi *et al.* induced the differentiation of HSCs into thymocytes (precursor to T cells) via the presentation of surface-bound Notch ligand or delta-like ligand 4 (DLL4), using microbeads decorated with DLL4 to activate Notch signaling, although this protocol still required co-culture with stromal cells (OP9 cell line) (82).

1.4.3. Matrix Cues on HSC Fate Decisions

As previous attempts to expand or differentiate HSCs with the addition of cytokines have been largely unsuccessful without the use of feeder cells, using niche-mediated microenvironmental or biophysical cues to engineer HSC fate decisions by mimicking physiological niche conditions has gained interest as a potential approach to engineer HSC fate decisions (83). In 2006, Engler *et al.* showed successful differentiation of MSCs to osteogenic, myogenic, and neurogenic lineages using niche-mimicking, compliant substrates as a culture platform in otherwise identical normal growth culture medium, highlighting the utility and the relevance of matrix cues (27). This breakthrough spurred hope that fate decisions of other adult stem cells such as HSCs could also be controlled *in vitro* in a similar fashion. However, while matrix mechanics has become a primary design principle for a range of other stem cell
populations (e.g., MSCs (27), embryonic stem cell (ESC) (84), neural stem cells (NSC)(85)) , the impact of matrix mechanics on HSC fate remains poorly explored (2, 3). Notably, HSCs can quickly lose long-term repopulating activity and go through excessive differentiation and/or apoptosis in vitro (11-13). While this latter issue may be solved via temporal control of cytokine administration as reported by Csaszar et al. in 2012 (86), investigating the impact of matrix biophysical signals on HSCs remains a significant open question. Consequently, despite significant information regarding biochemical signals (e.g., cytokines, chemokines, growth factors) impacting HSC fate currently no biophysical explanation exists for the niche-mediated regulation of HSC proliferation and lineage specification.

As described in §1.2, HSCs reside in a specialized niche that comprises of ECM components, soluble and immobilized cytokines, growth factors, and chemokines, and various hematopoietic and non-hematopoietic cell types, forming a complex, spatially gradated microenvironments across the marrow (1-3, 8, 9). Due to variable composition in biochemical and biophysical content and biomechanical forces across the marrow, it is not possible to fully recapitulate a continuum of marrow niche microenvironments in vitro with currently available techniques. Further, the niche components essential for reliably guiding HSC fate decisions are yet to be established. Nonetheless, microenvironmental gradations across the marrow (23, 26) and the localization of specialized HSC niches within and across these spatial regions (25) suggest that biophysical or matrix cues may provide important extrinsic signals to regulate HSC fate decisions. Indeed, previous reports support this notion. First, several ECM proteins such as fibronectin (87-89), collagen (90), laminin (88, 91), and MMPs (29, 92) have been implicated with modulating HSC functions and/or hematopoiesis. Notably, fibronectin has been shown to promote long-term maintenance and expansion of HSCs in vitro (87-89). Second, varying
subsets of hematopoietic stem and progenitor cells have been shown to respond to changes in substrate elasticity (93-95), topography (96-98), and lateral spacing of ligands (99, 100). For instance, Holst et al. showed that human and mouse whole bone marrow cells cultured on tropoelastin-coated substrates result in significant enrichment in HSC fractions compared to bare tissue culture plates due to changes in substrate elasticity (93). Similarly, culturing HSCs on eletrospun polyethersulfone nanofibers (98) or microcavities with a diameter of 15-μm or smaller (96, 97) led to increased expansion due to changes in substrate topography. In 2012, we showed (Choi et al.) that spread area and cytoskeletal organization of HSCs increase when they are cultured on stiffer collagen-coated substrates, with similar changes observed in 3D collagen gels with varying stiffness (94). These observations imply matrix-mediated regulation of HSC fate, but its impact on specific HSC fate decisions, particularly regarding quiescence vs. self-renewal, self-renewal vs. lineage specification, and downstream development into mature cell types in relation to niche microenvironmental conditions still need to be investigated.

1.5. *In vitro* Culture Platforms for HSCs

Our understanding of the matrix-mediated regulation of HSC fate decisions is currently limited because niche composition, particularly those niche components essential for guiding specific HSC fate decision processes, remains uncertain. Nonetheless, we propose to leverage known information regarding the marrow microenvironments to generate ‘building bocks’ of an *in vitro* culture platform, with the goal to examine synergies between elements of an engineered marrow to identify a pathway to engineer HSC fate decisions (Fig. 1.5). This effort is inspired by recent success of an *in vivo* bottom-up approach recently described by Calderon et al. using Foxn1-deficient (*Foxn1*−/−) nude mice whose thymus lacks hematopoietic capacity to create
artificial thymopoietic environments in the mouse embryo (101, 102). Because hematopoietic activity of the thymus (e.g., secretion of relevant chemokines, cytokines, and Notch ligands) depends on the transcription factor Foxn1, Calderon et al. were then able to selectively turn on the expression of Ccl25, CXCL12, SCF, DLL4, or combinations thereof (‘building blocks’) using a Foxn1 promoter to re-functionalize a defective thymic epithelium. This effort showed that approaches that aim to build layers of complexity into an experimental platform have the potential to generate significant new insight regarding important regulatory circuits within a complex signaling network. Our approach is inspired by this effort. Here we integrate cytokines and matrix cues as our ‘building blocks’ to create artificial HSC niche microenvironments within an in vitro culture platform (Fig. 1.5). This approach would be ideal for studying or engineering HSC fate decisions while dynamically monitoring any functional changes to integrate feedback immediately. Design criteria for such a platform would include the following: 1) ease of fabrication, 2) combinatorial in nature to incorporate several different cues, 3) scalable to study small numbers of rare populations or to scale-up for large scale production if necessary, 4) in situ analysis of cells at the single cell resolution possible, 5) optionally, high-throughput analysis possible. Several groups have attempted to build an in vitro platforms using similar approaches, based primarily on microfluidic (e.g., microwells, gradient mixer) (103-105) or microarray techniques (106). Notably, our group (Mahadik et al.) designed a microfluidic-based gradient mixer for creating small volumes of 3D hydrogels with opposing gradients of cells and/or matrix components (105). However, these platforms do not satisfy all of the design criteria, particularly with regards to in situ analysis of single cells. This type of in situ analysis to accompany in vitro culture platforms is desirable because it provides a unique advantage over other in vivo models (57). Also, it could be used to resolve heterogeneity, which has gained attention recently as
heterogeneity within cell populations such as cancer cells (107) or stem cells (e.g., HSCs (55)) has been linked to significant differences in functional output among sub-populations. This issue of heterogeneity is especially problematic for HSCs because putative HSCs often form an inherently heterogeneous cell population unless very rigorous purification scheme is used (108). The rarity and uncertainty associated with identifying bona fide HSCs and the lack of functional assays for HSC self-renewal or differentiation at the single cell level exacerbates this problem. Therefore, our approach would include testing new single cell metrics for in situ analysis of HSCs being cultured on our in vitro platform (Fig. 1.5). For this, we will assess the feasibility of traditional staining approaches (e.g., immunofluorescence) as well as new techniques (e.g., time-of-flight secondary ion mass spectroscopy (TOF-SIMS), atomic force microscopy (AFM), Raman Spectroscopy, photonic crystal enhanced microscopy (PCEM)) to establish new, functional, single-cell metrics of HSCs.

The rest of this dissertation will delineate our effort to achieve this goal:

- In **Chapter 2**, the effect of marrow-inspired biophysical cues on HSC spreading and cytoskeletal reorganization in vitro will be discussed.

- In **Chapter 3**, the impact of context-driven matrix cues on HSC fate decisions, notably proliferation vs. lineage specification, will be described.

- In **Chapter 4**, the feasibility of single cell analysis methods (AFM, TOF-SIMS, Raman Spectroscopy) as functional metrics of hematopoietic stem and progenitor cells at the single cell level will be investigated.

- In **Chapter 5**, the use of adhesive signatures as a potential single cell metrics of HSC fate decisions will be outlined.
Finally in **Chapter 6**, I will present a summary of the findings from this thesis as well as present options regarding future experimental directions and opportunities.
1.6. Figures

Figure 1.1. Hematopoiesis scheme.
HSCs mediate life-long hematopoiesis to supply the full complement of blood and immune cells. During hematopoiesis, HSC fate decision processes are tightly controlled to maintain a constant pool of stem and progenitor cells and differentiated progeny. (LT-HSC: Long-Term HSCs; ST-HSC: Short-Term HSCs; MPP: Multipotent Progenitors; CMP: Common Myeloid Progenitors; CLP: Common Lymphoid Progenitors.)
Figure 1.2. Structure and composition of the bone marrow.
Bone marrow is a complex tissue with spatial and dynamic variations in biophysical cues, biomolecular cues, local cellular composition, and marrow vasculature across the marrow.

Images modified from: 
- Morrison, Scadden (2014) (109)
- Urbach Lab
  (http://www.physics.georgetown.edu/~urbach/research.html)
- BioNinja Biology Resource
- Deltagen, Inc.
  (http://www.deltagen.com/target/histologyatlas/atlas_files/hematopoietic/bone_marrow_40X.jpg)
Figure 1.3. Blood supply of the bone marrow.
The artery first enters the cortical bone to split into ascending and descending branches that run axially along the central marrow cavity, then radially towards the endosteal region while giving rise to a network of smaller arterioles (Sca-1⁺) and capillaries that anastomose with wider, irregularly-shaped venous sinusoids (Sca-1⁻).

Images modified from:  
Travlos (2006) (22)  
Wideman, Prisby (2012) (110)  
Nombela-Arrieta et al. (2013) (25)  
McGraw-Hill Companies, Inc.  
(http://www.mhhe.com/biosci/esp/2002_general/Esp/folder_structure/tr/m3/s8/trm3s8_2.htm)
Figure 1.4. Extrinsic control of HSCs.
Extrinsic control of HSC fate decisions entails the use of soluble factors (cytokines, chemokines, growth factors), niche cells, and marrow-inspired matrix cues.
Figure 1.5. Schematic of an *in vitro* biomaterials culture platform.
Extrinsic control of HSC fate decisions in an *in vitro* platform requires the addition of suitable ‘building blocks’ (soluble factors, niche cells, matrix cues) and the capacity to continuously analyze cells *in situ*.

Image modified from: *Chen et al.* (2013) (111)
1.7. Tables

Table 1.1. ECM protein expressions across the marrow.
ECM protein expressions across the marrow show spatial variations.

Table modified from: *Nilsson et al.* (1998) (26)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Endosteum</th>
<th>Central Marrow</th>
<th>Marrow Vessels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibronectin</td>
<td>+++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Collagen I</td>
<td>+++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Collagen II</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Collagen IV</td>
<td>++</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Laminin</td>
<td>-</td>
<td>+</td>
<td>+++</td>
</tr>
</tbody>
</table>
Table 1.2. Fractions of hematopoietic progenitors in the marrow.
Frequency of varying subsets of hematopoietic stem and progenitors in the bone marrow.

Data compiled from: *Kiel et al. (2005)* (45)  
*Schroeder (2010)* (56)  
*Grassinger et al. (2010)* (112)

<table>
<thead>
<tr>
<th>Cell Surface Antigen Expression</th>
<th>Frequency (% BM)</th>
<th>Estimated frequency of <em>bona fide</em> HSCs within population (%) based on engraftment studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>LT-HSC CD34-LSK</td>
<td>~0.005*</td>
<td>30-40</td>
</tr>
<tr>
<td>ST-HSC CD34+LSK</td>
<td>~0.045*</td>
<td>--</td>
</tr>
<tr>
<td>HSC CD150+CD244-CD48-</td>
<td>0.01</td>
<td>--</td>
</tr>
<tr>
<td>HSPC LSK</td>
<td>~0.05</td>
<td>~3</td>
</tr>
<tr>
<td>MPP CD150-CD244+CD48-</td>
<td>0.19</td>
<td>--</td>
</tr>
</tbody>
</table>

*Experimentally determined*
CHAPTER 2: Impact of Matrix Elasticity, Construct Dimensionality, and Ligand Density on the Viability and Biophysical Properties of Hematopoietic Stem and Progenitor Cells

2.1. Abstract

Hematopoietic stem cells (HSCs) are adult stem cells with the capacity to give rise to all blood and immune cells in the body. HSCs are housed in a specialized microenvironment known as the stem cell niche, which provides intrinsic and extrinsic signals to regulate HSC fate: quiescence, self-renewal, differentiation, mobilization, homing, and apoptosis. These niches provide a complex, three dimensional (3D) microenvironment consisting of cells, the extracellular matrix (ECM), and ECM-bound or soluble biomolecules that provides cellular, structural, and molecular signals that regulate HSC fate decisions. In this study, we examined the decoupled effects of substrate elasticity, construct dimensionality, and ligand concentration on the biophysical properties of primary hematopoietic stem and progenitor cells (HSPCs) using homologous series of two and three dimensional microenvironments. Microenvironments were chosen to span the range of biophysical environments presented physiologically within the bone marrow, ranging from soft marrow and adipose tissue (< 1 kPa), to surrounding cell membranes (1~3 kPa), to developing osteoid (>30 kPa). We additionally investigated the influence of collagen ligand density on HSPC biophysical parameters and compared these behaviors to those observed in HSPCs grown in culture on stiff glass substrates. This work suggests the potential for substrate stiffness and ligand density to directly affect the biophysical properties of primary

---

1 This chapter has been adapted from the following publication: Choi JS & Harley BAC (2012) The combined influence of substrate elasticity and ligand density on the viability and biophysical properties of hematopoietic stem and progenitor cells. *Biomaterials* 33(18):4460-4468.
hematopoietic stem and progenitor cells at the single cell level and that these parameters may be critical design criteria for the development of artificial HSC niches.

2.2. Introduction

Hematopoietic stem cells (HSCs) are adult stem cells that reside primarily in the bone marrow with the capacity to give rise to all blood and immune cells in the body. A hierarchical system of HSCs and progenitor cells exists, differing in their ability to reconstruct the bone marrow. HSCs are housed in a specialized microenvironment known as the stem cell niche, which provides intrinsic and extrinsic signals to regulate HSC fate: quiescence, self-renewal, differentiation, mobilization, homing, and apoptosis (1, 113). During homeostasis, quiescent HSCs are thought to primarily localize near the endosteum (‘endosteal niche’), a bone marrow microenvironment lined by osteoblasts and nestin-expressing mesenchymal stem cells (MSCs) that supply factors (i.e. CXCL12, SCF, Ang-1, VACM-1, and TPO) essential for HSC maintenance (114). More active, cycling HSCs are thought to be primarily located in close proximity to vascular surfaces (‘perivascular niche’) where they are associated with CXCL12-abundant reticular (CAR) cells, perivascular MSCs, and sinusoidal endothelium (114). These niches provide a complex, three dimensional (3D) microenvironment consisting of cells, the extracellular matrix (ECM), and ECM-bound or soluble biomolecules that provides cellular, structural, and molecular signals that regulate HSC fate decisions (1, 114). However, both the critical elements of this niche and the role they play on directing HSC fate decisions remain a significant open question.

Matrix elasticity and durotaxis mechanisms are strong regulators of stem cell fate (27, 84, 115). The role played by biophysical properties on stem cell fate decisions has been a topic of
great interest over the last decade. In the literature, a multitude of studies, primarily utilizing MSCs or embryonic stem cells (ESCs) have suggested a critical role played by matrix mechanical properties in determining stem cell fate. Perhaps most famous is seminal work by Engler et al. (27), who showed that human MSCs were found to selectively initiate neurogenic, myogenic or osteogenic differentiation pathways when they were cultured on collagen-coated polyacrylamide (PA) substrates with varying stiffness mimicking the stiffness of the brain (0.1 – 1 kPa), muscle (8 – 17 kPa) or the collagenous bone (25 – 40 kPa). Similarly, myogenic commitment of MSCs was found to be differentially regulated by both substrate stiffness and substrate chemistry (116). In murine ESCs, optimal spreading was observed on soft substrates (0.6 kPa) while more differentiated cells derived from these ESCs spread significantly more with increasing substrate stiffness (84). Substrate modulus was also found to regulate neural stem cell behavior, with soft substrates (100 – 500 Pa) favoring neural differentiation and stiff substrates (1 – 10 kPa) inducing glial cultures (85).

While the role played by cell-cell interactions regulating HSC fate decisions (i.e. quiescence, differentiation, self-renewal) has been an important area of research (1, 113, 114), the role played by the biophysical environment (mechanics, microstructure) on HSC fate regulation has only recently started to be explored. Holst et al. observed that the HSC population (Lin- Sca-1^+ c-Kit^+) within a culture of mouse whole bone marrow cells were enriched up to 14.2-fold when they were cultured on tropoelastin-coated well-plates. This effect was not seen when the mechanosensing ability of the cells were masked by inhibiting myosin II heavy chain (blebbistatin) and myosin light chain kinase (ML-7) or when the stiffness of the tropoelastin network was significantly increased via glutaraldehyde crosslinking; these results highlighted the potential for substrate tensegrity to play a role in HSC regulation (93). However, because these
cultures were performed using whole bone marrow, it remains unclear as to whether the effect of substrate mechanics on HSC fate is direct or due to effects of substrate stiffness on some or all of the other cell populations within whole bone marrow which includes known mechano-sensitive MSCs. Other studies have reported several integrins and matrix proteins (i.e., fibronectin, laminin, collagen I, etc.) as mediators of HSC fate using primary HSCs and cell lines (88, 96, 97, 99, 117, 118).

In this study, we aim to decouple the effects of substrate elasticity, ligand concentration, and dimensionality on HSC fate by culturing primary HSCs in or on top of collagen hydrogels or on top of 2D PA substrates with varying mechanics and ligand densities. Physiological environments of the HSC niches exhibit a range of stiffnesses, ranging from soft marrow (< 1 kPa) to adipose tissue (1~3 kPa) to non-mineralized bone (> 34 kPa) (23, 27, 119). With collagen hydrogels and type I collagen-coated PA substrates with varying stiffness, we created simple in vitro biomaterials system to probe the effects of substrate mechanics on the biophysical properties of HSCs. In order to compare the behavior of these primary HSCs with a stable cell population, 32D cells were used as a control. 32D cells are interleukin-3 (IL-3) dependent murine myeloid progenitor cells that are widely used as an in vitro model of hematopoiesis (120, 121). They terminally differentiate into neutrophilic granulocytes upon the removal of IL-3 and injection of granulocyte-colony stimulating factor (G-CSF) to the culture medium, and this differentiation process closely resembles the differentiation patterns inside the marrow (120, 121).

2.3. Materials and Methods
2.3.1. Preparation and collagen functionalization of 2D polyacrylamide substrates

Polyacrylamide (PA) substrates with tunable substrate elasticity were fabricated from defined ratios of acrylamide and bis-acrylamide (Fisher Scientific, Hampton, NH) in the microwell of 14-mm glass bottom culture dishes (MatTek Corp., Ashland, MA) according to previously described methods (122, 123). Briefly, the glass was silanized with 3-aminopropyltrimethoxysilane (Fisher Scientific, Hampton, NH) and a solution containing defined ratios of acrylamide:bis-acrylamide, ammonium persulfate (1/200 v/v, Fisher Scientific, Hampton, NH), and tetramethylethylenediamine (1/2,000 v/v, Fisher Scientific, Hampton, NH) was deposited. A 12-mm circular coverslip (Fisher Scientific, Hampton, NH) was placed on top of the mixture and the dish was placed upside down in a humidified incubator at 37 °C to ensure formation of an even, consistent surface. After polymerization the coverslip was carefully removed and the PA substrate was rinsed thoroughly with 50 mM HEPES buffer, pH 8.5 (MediaTech, Inc., Manassas, VA). Completed PA substrates were then stored in DI water at 4 °C until needed. For this investigation, four distinct PA substrate variants were fabricated with defined acrylamide (v/v %):bis-acrylamide (v/v %) ratios: 4%/0.03%, 5%/0.1%, 5%/0.26%, and 38%/2%. These ratios were chosen so as to present a range of substrate elastic moduli between 1 and 200 kPa (23, 27, 124).

Prior to use, the PA substrates were coated with defined concentrations of type I collagen using previously described methods (84, 124). The PA substrates were first functionalized with Sulfo-SANPAH (Fisher Scientific, Pittsburgh, PA) via UV exposure (365 nm) and then rinsed with 50 mM HEPES. Diluted (100 μg/mL, 40 μg/mL, or 1 ng/mL) type I collagen solutions were prepared with the identical stock collagen solution used to create 3D collagen hydrogels (BD Biosciences, Bedford, MA), diluted in 50 mM HEPES. Collagen dilutions were chosen from
those previously used to investigate embryonic stem cell biophysical properties (84). The diluted collagen solution was then placed on top of the sulfo-SANPAH activated PA substrates and left to react overnight at 4 °C. Coated PA substrates were rinsed the next day and stored in DI water at 4 °C for up to two days until use. As controls, bare glass MatTek dishes (glass(col-)) and MatTek dishes where the glass was directly functionalized with 100 μg/mL type I collagen (glass(col+)) were also prepared. Prior to cell seeding all substrates were sterilized with UV and allowed to equilibrate in the corresponding culture medium for at least 30 minutes.

2.3.2. 3D collagen hydrogel preparation

Collagen hydrogels were fabricated with type I collagen from rat tail (BD Biosciences) in accordance with previously described methods (125, 126). In brief, the collagen stock solution was diluted with culture medium to a final concentration of 1.45 or 2.9 mg/mL and buffered to a pH 7.4 with 1 M HEPES buffer and 0.4 M NaOH. All reagents for collagen hydrogel fabrication were pre-cooled to 4 °C to prevent any premature collagen gelation upon mixing. The collagen solution was then placed in a 14-mm dia. glass bottom dish (MatTek) and was incubated in a humidified 37 °C incubator to complete polymerization. To encapsulate cells within the 3D collagen hydrogel constructs, a cell suspension in the equivalent volume of cell media was added to the stock collagen solution in lieu of culture media alone. To culture cells on top of the 3D collagen hydrogels, the hydrogels were allowed to completely polymerize (2 hours) prior to cell seeding in a manner identical with the 2D PA substrates.
2.3.3. Mechanical characterization

2.3.3.1. Atomic force microscopy

Elastic moduli of uncoated PA and collagen-coated PA substrates was determined via atomic force microscopy (AFM; MFP-3D, Asylum Research Santa Barbara, CA) from multiple force-indentation curves for each PA variant (n = 30 - 45) in a manner previously described (27, 127, 128). Briefly, each substrate was pre-saturated in deionized water. A silicon nitride probe with a pyramidal tip and a nominal spring constant of 0.06 N/m (Bruker Corp., Camarillo, CA) was used to collect force-indentation profiles that were analyzed with a Hertz cone model to compute the elastic moduli of the corresponding PA substrates (128). The Poisson ratio (ν) was assumed to be 0.4 for these calculations, consistent with the published range of 0.3 – 0.5 for PA substrates (127). Elastic moduli were calculated for three of four experimental PA substrates: 5%/0.1%, 5%/0.26%, and 38%/2% (acrylamide/bis-acrylamide (v/v %)). These values were well-correlated with previously reported calibration curves (124) so subsequent PA substrate stiffnesses were reported as interpolated values. To assess the effect of collagen-coating density on PA substrate stiffness, the elastic moduli of the 38%/2% PA substrate was additionally determined for each of the three experimental collagen densities (100 μg/mL, 40 μg/mL, or 1 ng/mL).

2.3.3.2. Rheology

The elastic moduli of the 3D collagen hydrogels were determined with a Bohlin C-VOR rheometer (Malvern Instruments, Westborough, MA) in oscillatory mode with a fixed frequency of 1 Hz and an applied strain of 1% (125, 126). A small volume of each collagen hydrogel solution was placed on the rheometer stage held at 25°C to prevent premature gelation and was
then tested using a 4° 20-mm cone with a solvent trap. Briefly, the stage temperature was rapidly raised to 37 °C to induce faster (system-relevant) polymerization; three-minute oscillatory tests were then performed at twenty-minute intervals for a total of 12 hours to determine the collagen gelation kinetics and long term stability for the duration of the HSPC bioactivity studies. The elastic moduli of each hydrogel was then determined from the storage modulus from the results following the completion of gelation.

2.3.4. Hematopoietic stem and progenitor cell isolation and culture

HSPCs were isolated from the femurs and tibias of 4 – 10-week-old C47Bl/6 mice (The Jackson Laboratory, Bar Harbor, ME). Briefly, mice were euthanized with carbon dioxide in compliance with the University of Illinois Institutional Animal Care and Use Committee (IACUC) guidelines. Their femurs and tibias were then collected, crushed, and filtered through a 40-μm cell strainer (BD Falcon, Franklin Lakes, NJ) to yield a whole bone marrow cell suspension in PBS supplemented with 2% FBS. Red blood cells were lysed using ACK lysis buffer (Invitrogen, Carlsbad, CA) and the resulting suspension was then incubated with Fc receptor blocking antibody (CD16/CD32) to prevent nonspecific antibody binding. The cells were then incubated with a cocktail of FITC-conjugated lineage (Lin) antibodies (CD5, CD45R (B220), CD11b, Anti-Gr-1 (Ly-6G/C), 7-4, Ter-119), PE-conjugated Sca-1, and APC-conjugated c-Kit. (All antibodies purchased from eBiosciences, San Diego, CA). The labeled suspension was sorted via fluorescence-activated cell sorting (FACS) using BD FACSria™ cell sorter for the Lin’Sca-1+c-Kit+(LSK) HSPC population (1). Dead cells were excluded with propidium iodide (PI) staining. Sorting consistently yielded 4E4 – 6E4 HSPCs per mouse (Fig. 2.1), corresponding to ~0.5% of whole bone marrow and consistent with previously published reports
(1). All steps of the HSPC isolation process were performed in PBS + 2% FBS until LSK cells were sorted into a previously defined HSPC culture media containing serum-free Stemline® II hematopoietic stem cell expansion medium (Sigma, St. Louis, MO) supplemented with 100 ng/mL SCF (Stem Cell Technologies, Vancouver, Canada) and 4 ng/mL Flt-3/Flk-2 ligand (Stem Cell Technologies Vancouver, Canada) (103).

Once isolated, LSK HSPCs were then immediately seeded at a density of 7,000 – 8,000 cells/sample in 150 µl HSPC culture media onto 2D PA substrates or onto previously polymerized 3D collagen hydrogels. Additional culture media was placed after an hour to ensure cell attachment. Alternatively, 7,000 – 8,000 LSK HSPCs were mixed with type I collagen, culture media, HEPES, and NaOH in order to encapsulate HSPCs within the 3D collagen hydrogel network. The cells were incubated in HSPC culture media in a humidified incubator at 37°C with 95% oxygen and 5% CO₂ supply for 24 hours.

2.3.5. Culture of 32D cells

The IL-3 dependent murine myeloid progenitor cells line (32D) has been widely used as an in vitro model to study hematopoietic cell behaviors including proliferation, differentiation, and apoptosis (121), and were used here as a control for comparison to HSPC behavior. The 32D clone 3 (32D Cl3) cell line was purchased from ATCC (Manassas, VA,) and was subcultured according to ATCC protocols in a complete growth medium prepared from RPMI 1640 medium, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, 2 mM L-glutamine, 10% heat inactivated FBS, and 10% mouse IL-3 (BD Sciences, Franklin Lakes, NJ). Like HSPCs, 7,000 – 8,000 32D cells were either seeded on top of PA substrates or 3D collagen
hydrogels or were encapsulated within 3D collagen hydrogels. 32D cells in each constructs were incubated in a humidified incubator at 37°C with 95% oxygen and 5% CO₂ for 24 hours.

2.3.6. Viability assays

HSPC and 32D cell viability was assessed after 24 hours of culture, using the LIVE/DEAD Cell Viability Assay (Invitrogen, Carlsbad, CA). After staining the cells, each construct was imaged at a minimum of 5 locations using an inverted fluorescence microscope (DMI4000, Leica Microsystems, Germany) with a 40x objective. The relative numbers of live (calcein positive) versus dead (ethidium homodimer-1, red) cells was then averaged to obtain the mean cell viability of each corresponding sample.

2.3.7. Immunofluorescence staining and confocal imaging

After 24 hours of culture, HSPCs and 32Ds were fixed with 3.7% paraformaldehyde, permeabilized with 0.1% Triton® X-100 (Fisher Scientific, Hampton, NH), and then sequentially incubated with Image-iT® FX signal enhancer (Invitrogen, Carlsbad, CA), Alexa Fluor® 488-conjugated phalloidin (to stain F-actin filaments), and DAPI (to label cell nuclei). The sample was rinsed thoroughly with PBS three times between each step. All reagents were purchased from Invitrogen (Carlsbad, CA) unless noted otherwise. ProLong® Gold antifade reagent (Invitrogen, Carlsbad, CA) was then applied and samples were subsequently stored in the dark at 4°C in order to minimize sample photobleaching. Fixed samples were imaged using an LSM 710 (Carl Zeiss AG, Thornwood, NY) multi-photon laser scanning microscope using a 63x objective (N.A. = 1.4) and the Zeiss Zen software (Carl Zeiss AG, Thornwood, NY) to obtain 3D images of the cells.
2.3.8. Image analysis of cell size, shape, and cytoskeletal organization

2.3.8.1. Cell spread area and cell shape index (CSI)

The spread area and cell shape index (CSI) of the cells cultured on 2D substrates were calculated via ImageJ from projected 2D images of the cells gathered with confocal microscopy. Briefly, the cell images were thresholded and individual cells were outlined in order to compute the spread area and the perimeter of the cells. CSI, a dimensionless measure of the circularity of a cell \((4 \cdot \pi \cdot \text{(cell area)} \cdot \text{(cell perimeter)}^2)\), has previously been used to assess biophysical properties of a range of cell types (129). CSI values are reported on a continuum between 0 (theoretical minimum for cell infinitely elongated in one axis) and 1 (perfectly circular cells). Single cells in direct contact with other cells or undergoing mitosis were excluded from analysis so as to assess the effect of substrate properties alone.

2.3.8.2. Cytoskeletal density across a cell

The radial distribution of cytoskeletal elements within the HSPCs was determined using a previously described method from projected 2D images of 3D image stacks collected via multi-photon microscopy (84). Three arbitrary radial lines were drawn from an arbitrary center towards the edge of each cell; the F-actin fluorescence intensity along each radial line through the cell cytoplasm was quantified using AxioVision (Carl Zeiss AG, Thornwood, NY). For most cells, a low value of CSK intensity was observed near the nucleus while a subsequent spike in the fluorescence intensity was observed towards the outer edge of the cells. The normalized width \((x)\) and height \((y)\) of this increase in CSK intensity was calculated for all cells cultured on 2D substrates to elucidate the relationship between substrate stiffness and cytoskeletal distribution.
These absolute fluorescence values were normalized by the radius of the cell (x) and the baseline fluorescence intensity values outside the cell to account for differences in cell size with substrate elasticity as well as the potential for differences in fluorescence intensity observed experiment to experiment.

### 2.3.9 Statistical analysis

One-way analysis of variance (ANOVA) with Tukey post-hoc testing was performed in OriginPro (Origin Lab Corp., Northampton, MA) to determine statistical differences between groups. For all figures, values are reported as mean and standard error of the mean (SEM) unless otherwise noted. Mean and standard deviation were reported for the PA substrate elastic moduli values, consistent with previous reports (124).

### 2.4. Results

#### 2.4.1 Mechanical characterization of collagen hydrogels

Rheological measurements of the collagen hydrogels revealed they polymerized rapidly (within the first two hours). The elastic moduli of the collagen hydrogels remained stable once this initial polymerization phase was complete (data not shown). The elastic moduli of the two collagen hydrogel concentrations used in this experiment (1.45 mg/mL; 2.9 mg/mL) were found to be significantly \( p < 0.05 \) different: 0.0148 ± 0.0061 kPa and 0.0442 ± 0.0107 kPa, respectively.
2.4.2. Mechanical characterization of polyacrylamide (PA) substrates

AFM analysis showed the elastic moduli of the uncoated PA substrates increased with increasing acrylamide/bis-acrylamide (v/v %) concentrations, 5%/0.1%, 5%/0.26%, and 38%/2% (Table 2.1). These values ranged from 3.5 to 196 kPa and correlated well with previously reported values (123, 124, 127); thus, all other PA substrates fabricated were assumed to hold moduli as published. In summary, the PA substrates we created had Young’s moduli ranging from 0.71 kPa to 196 kPa (Table 2.1; Fig. 2.2(A)).

The surface density of collagen conjugation to the PA substrate surface was quantified via immunofluorescence, showing collagen surface densities ranging between 0.024 μg/cm² – 1.36 μg/cm² (Fig. 2.2(B)). For simplicity, throughout the rest of the manuscript collagen ligand density will be reported as the amount of collagen dissolved in DI water that was used to create the collagen-modified surfaces (1 ng/ml, 40 μg/ml, 100 μg/ml). The effect of collagen conjugation and collagen-coating density on PA substrate stiffness was assessed by comparing the elastic moduli of the same PA substrate (196 ± 54.1 kPa) left uncoated with those coated with three different collagen densities (1 ng/ml, 40 μg/ml, 100 μg/ml). There were no statistical differences (p > 0.05) between uncoated and coated samples, as well as between samples coated with varying collagen concentrations (data not shown). As collagen coating or coating density had a negligible effect on substrate elasticity, for all analyses in this manuscript we will report substrate stiffness values as those for the uncoated PA substrate variants.

2.4.3. HSPC isolation

Lin-Sca-1+c-Kit+ hematopoietic stem and progenitor cells (HSPCs) were isolated from murine bone marrow via FACS. Sorting consistently yielded 4E4 – 6E4 HSPCs per mouse (Fig.
2.1), corresponding to ~0.5% of whole bone marrow and consistent with previously published reports (130).

2.4.4. *Effect of substrate dimensionality on HSPC biophysical properties*

After 24 hours of culture, HSPCs seeded on top of (2D) or encapsulated within (3D) collagen hydrogels showed hydrogel density and dimensionality related effects. The effect of dimensionality, although subtle, was observed in terms of cell viability and the level of cytoskeletal organization. Regardless of hydrogel density (1.45 and 2.9 mg/mL), HSPCs cultured within the hydrogel constructs showed decreased cell viability compared to those cultured on top of the same constructs, though the effects were not significant (Fig. 2.3(A)). However, suggestive trends were observed in cell morphology (shape, size) for HSPCs cultured on top of collagen or within the collagen hydrogel constructs (Fig. 2.3(B)). Qualitatively, HSPCs cultured on top of or inside stiffer collagen hydrogels (2.9 mg/mL; 0.0442 ± 0.0107 kPa) showed more cytoskeletal development or cytoplasmic protrusions compared to those grown on top of or inside softer collagen hydrogels (1.45 mg/mL; 0.0148 ± 0.0061 kPa) as evidenced by larger and denser cytoplasmic area observed via confocal imaging. Further, examining the projected area of the HSPCs cultured on top of vs. within the constructs, HSPCs cultured in 2D environments appeared more spread out compared to those encapsulated in 3D, which retained a more rounded morphology. In stiffer 3D constructs (2.9 mg/mL), HSPCs started developing thin, filopodial protrusions forming at the cytoplasmic region enriched in F-actin fibers (Fig. 2.3(B)), although they still maintained overall more rounded morphology compared to those grown on top of 2D equivalents.
2.4.5. Effect of substrate stiffness on HSPC viability and morphology

More significant differences in HSPC biophysical properties were observed after culturing them on a homologous series of 2D collagen substrates for 24 hours (Substrate moduli: 14Pa – 196kPa; Collagen coated glass control) (Fig. 2.4). HSPC viability was significantly ($p < 0.05$) influenced by substrate stiffness. HSPC viability for 0.0442 kPa substrates was significantly higher ($p < 0.05$) than 3.48 kPa PA substrates and uncoated glass control samples while being non-significantly greater ($p \leq 0.8$) than other substrates (Fig. 2.4(A)). Lowest HSPC viability was observed on non-coated glass slide controls, though no significant difference ($p = 0.9$) was observed between collagen coated (100 µg/ml; glass (col+)) and uncoated (0 µg/ml; glass (col-)) glass slides.

More substantial differences were observed in HSPC size and shape after culture on collagen coated substrates of varying substrate elasticity (Fig. 2.4(B)). When cultured on top of the collagen hydrogel substrates (0.0148, 0.0442 kPa), HSPCs spread more on the stiffer hydrogel. Similarly, a significant influence ($p < 0.05$) of PA substrate stiffness was also observed on HSPC spreading. Within this homologous series of substrates (collagen coating density: 100 µg/mL), no difference in HSPC spreading ($p > 0.05$) was observed on substrates with moduli on the order of individual cell membranes (0.71 – 8.95 kPa). However, HSPC spreading was observed to increase significantly ($p < 0.05$) for stiffer substrates (196 kPa PA), with significantly greater spreading ($p < 0.001$) observed on collagen-coated (100 µg/mL) glass control cultures. This effect was stiffness and ligand-density specific, as HSPC spreading on non-coated glass slide controls was significantly ($p < 0.001$) reduced. Substrate stiffness and ligand density also appeared to have some effect on cell shape, though not as pronounced as the effect on cell spreading. HSPCs showed more noncircular morphology as the substrate stiffness
increased across the entire range of substrate stiffnesses tested, though the effect was mostly non-significant (3.48 kPa vs. glass(col+): $p < 0.05$); however, HSPCs were significantly ($p < 0.05$) more rounded in non-coated versus collagen-coated glass slides (Fig. 2.4(D)). Quantifying the cytoskeletal density as a function of HSPC radius (84), the fluorescence intensity of F-actin staining (a measure of cytoskeletal density) within the HSPC cytoskeleton increased with increasing substrate stiffness, though the change was not statistically significant (Fig. 2.5), possibly due to differences being masked by internal normalization of fluorescence intensity associated with this analysis.

2.4.6. Effect of substrate collagen density on HSPC biophysical properties

To specifically interrogate the influence of collagen ligand density on HSPC behavior, PA substrates with a constant modulus of 196 kPa were coated with one of three different collagen concentrations (1 ng/mL, 40 μg/mL, 100 μg/mL). A significant ($p < 0.05$) influence of substrate ligand density was observed on HSPC viability, size, and shape (CSI) (Fig. 2.6). A significant ligand density dependent decrease in HSPC viability was observed with decreasing ligand density (Fig. 2.6(A)). Similarly, HSPC spread area decreased significantly ($p < 0.05$) and cells became significantly ($p < 0.05$) more rounded, with decreasing collagen ligand density (Fig. 2.6(B), (C)).

2.4.7. Effect of substrate stiffness on the biophysical properties of 32D cells

For comparison, the influence of substrate stiffness and ligand density was also assessed on a myeloid progenitor cell line (32D) often used as a model HSPC cell (121). Not surprisingly, 32D cell viability was high (>80%) for all experimental conditions (data not shown), so only
changes in cell morphology (size, shape) is reported. Changes in 32D cell spread area and shape showed similar trends as those seen with the primary HSPCs (Fig. 2.7). 32D cells were in general much larger and more amorphous, with multiple elongated protrusions or high degrees of membrane ruffling (Fig. 2.7(B)). While not significant ($p > 0.05$), for both culture on collagen hydrogels (0.0148, 0.0442 kPa) as well as on the collagen coated (100 μg/mL) PA and glass substrates, 32D cells spread more with increasing substrate stiffness (Fig. 2.7(A)). Cell shape index ($CSI$) and substrate stiffness showed a different pattern (Fig. 2.7(C)). Although the overall trend was that the $CSI$ and substrate stiffness were related in an inverse fashion, the greatest $CSI$ was found for 0.71 kPa PA substrates instead of 0.0148 kPa hydrogels. $CSI$ for 0.71 kPa substrates was significantly higher ($p < 0.05$) than that for 3.48 and 196 kPa substrates. Overall, there was more variability in cell shape, as can be seen by greater error bars, and the cells were much more amorphous compared to HSPCs cultured on identical substrates. When exposed to disparate collagen ligand densities (1 ng/mL, 40 μg/mL, 100 μg/mL; $E_{\text{substrate}} = 196$ kPa), 32D cells again showed extremely high viabilities (>80%; data not shown), independent of ligand density. 32D cell morphology, however, was influenced by substrate ligand density, though in a manner slightly different than that seen for primary HSPCs. 32D cell spreading was significantly greater ($p < 0.05$) at the intermediate (40 μg/mL) ligand density (Fig. 2.8(A)); here 32D cells both spread most and cell morphology was significantly more amorphous ($p < 0.05$), as illustrated by a reduced cell shape index (Fig. 2.8(B)).

2.5. Discussion

HSCs lodged in the bone marrow are exposed to a complex, three dimensional (3D) microenvironment consisting of cells, the extracellular matrix (ECM), and ECM-bound or
soluble biomolecules (1). Extrinsic signals from the niche provide cellular, structural, and molecular signals that regulate HSC fate decisions (i.e., quiescence, self-renewal, differentiation, apoptosis) (131). Spatial and temporal heterogeneities within BM niches are particularly believed to differentially control HSC fate. Matrix and biomolecule based chemotactic, durotactic, and paratactic cues as well as cell-based paracrine and juxtacrine signaling pathways are all believed to play a role in HSC niche bioactivity; however their coordinated method of action remains unclear. The majority of the previous studies of HSPC-niche interactions have been largely limited to in vivo experiments with knockout or transgenic mice or liquid-based culture assays (103, 104, 132-136), and surprisingly little effort has been put in to decouple the effects of the mechanics and microstructure of the physical environment where HSPCs reside (1, 7, 113, 114, 137) until recently.

Holst et al (93) recently confirmed that substrate elasticity provides mechanical signals that regulate HSPC expansion. In this study, whole bone marrow cells seeded on tropoelastin-coated 6- or 24-well plates showed two- to three-fold expansion of undifferentiated HSPC subpopulations. Tropoelastin, which is the most elastic biomaterial known (138), was crucial in the increased expansion of HSPCs because truncated versions of tropoelastin or glutaraldehyde-treated tropoelastin-coated substrates did not reproduce this phenomenon. Also, the addition of specific inhibitors of myosin II heavy chain (blebbistatin) or myosin light chain kinase (ML-7) reversed the increased expansion of HSPCs, suggesting that tropoelastin promoted the self-renewal or proliferation of HSPCs through mechanostransduction. This finding, although a major first step in the elucidation of HSPC fate decision processes, was limited in the sense that it utilized whole bone marrow rather than an isolated HSPC population, making it unclear whether substrate elasticity directly or indirectly influenced HSPC fate. Further, while modulating
substrate stiffness via crosslinking, it did not consider the wide range of stiffnesses that HSPCs may experience in the bone marrow, notably the range covering adipose tissue \( (E_{\text{sub}_1} < 1 \text{ kPa}) \), cell membranes \( (E_{\text{sub}} = 1 – 3 \text{ kPa}) \), and collagenous bone (osteoid; \( E_{\text{sub}} = 100 \text{ kPa} \) \( (23, 27, 139) \).

PA substrates functionalized with matrix proteins such as collagen I and fibronectin have been employed in a number of cell-matrix interactions studies to elucidate the role of substrate mechanics on cell behavior for various stem (\( i.e., \) MSCs \( (27) \), ESCs \( (84) \), etc.) and more committed cell types \( (127, 140) \). Similarly, collagen hydrogels made solely from collagen or with other synthetic or natural biomaterials have been used widely to study cell interactions \( (126, 141) \). Because these systems are well-documented while providing a wide range of stiffness, these were chosen as cell culture platforms.

Currently, no well-established protocols exist for maintaining HSPCs viable in culture for a long period of time; optimization of such a culture environment requires design of the appropriate culture media as well as the biophysical environment in which culture takes place. In this project, we report the biophysical response of primary HSPCs cultured on a homologous series of 2D (collagen-coated) PA substrates and 3D collagen hydrogels with tunable mechanical properties. This experimental set-up allowed us to decouple matrix elasticity from other factors (growth factors, cell-cell interactions, etc.) in probing initial HSPC fate decisions. For this study, a 24 hour culture period was chosen to enable documentation of the initial changes in HSPC biophysical properties without worry of significant HSPC differentiation \( (142) \). Changes in initial HSPC viability, spread area, and cell shape provide insight on how HSPCs might behave in a microenvironment rich in certain components (\( i.e., \) adipose-rich bone marrow environment, \( E_{\text{sub}} < 1 \text{ kPa} \); perivascular environment rich with cell-cell contact, \( E_{\text{sub}} \sim 3 \text{ kPa} \); endosteal
microenvironment rich with osteon, $E_{\text{substrate}} \sim 100$ kPa). HSPC viability was enhanced at 0.0442 kPa (Fig. 2.4(A)), which suggests that HSPCs may initially attach to and survive better on softer substrates. However, HSPC viability was low across all experimental conditions, likely an artifact of the choice of a culture media chosen to not contain significant differentiation signals. As such, ongoing studies are looking to incorporate matrix-sequestered growth factors and cytokines in order to improve long-term HSPC growth.

Notably, we found that HSPC morphology (spreading, shape) was significantly influenced by the biophysical properties of the culture conditions alone. HSPC spreading, characterized by an increase in cell size and decrease in cell shape index, increased significantly with increasing substrate elasticity (Fig. 2.4(B)-(D)). When cultured on the stiffest PA substrates ($E_{\text{substrate}} = 196$ kPa), HSPCs spread out approximately twice as much as they did on substrates with an effective single cell stiffness ($E_{\text{substrate}} = 3.48$ kPa). HPSCs spread out even more on collagen-coated glass substrates ($E_{\text{glass}} \approx 70$ GPa; (143)), but HSPC spreading on bare glass was equivalent to the lowest degree of spreading observed (for the softest PA substrates). Overall, HSPCs retained a relatively rounded morphology, with $CSI > 0.6$ for all substrates (Fig. 2.4(D)). While not statistically significant, HSPCs became slightly more amorphous with higher degrees of membrane ruffling as substrate stiffness increased, corresponding with a drop in $CSI$. Again, HSPCs cultured on uncoated glass substrates maintained a significantly more circular morphology compared to those cultured on collagen-coated glass substrates that was equivalent to that seen with the softest substrates. Importantly, these results indicate that receptor-ligand interactions, as well as substrate elasticity, play an important role in co-modulating morphological changes in primary HSPCs during culture.
To further quantify the significance of receptor-ligand interactions on HSPC behavior, primary HSPCs were cultured on stiff PA substrates ($E_{sub} = 196$ kPa) coated with three different collagen coating concentrations (1 ng/mL, 40 μg/mL, 100 μg/mL). Viability was significantly impacted by substrate ligand presentation, with significantly fewer attached and viable HSPCs observed at the lowest (1 ng/mL) collagen-coating (Fig. 2.6(A)). HSPC spreading was also significantly impacted in a ligand density dependent manner; HSPC spreading decreased and HSPCs remained more rounded with decreasing collagen ligand density (Fig. 2.6(B), (C)). These results imply that HSPC binding through ECM proteins such as collagen is important in regulating HSPC fate. Substrate stiffness was also observed to impact HSPC cytoskeletal density and distribution, as measured by F-actin fluorescence intensity. Denser actin organization was observed at the leading edges of HSPCs cultured on stiffer substrates (Fig. 2.5). Such differences suggest that although actin fibers were punctate and did not form stress fibers within 24 hours of culture, the stiffness of HSPCs themselves may vary with substrate stiffness. Ongoing AFM-characterization across single HSPCs is expected to help resolve such information. Further, ongoing work is also examining the importance of HSPC-ligand interactions using a range of ECM proteins (fibronectin, laminin, vitronectin) as well as the use of further HSPC subfractions to determine whether subclasses of HSPCs show differential response to the divergent microenvironment cues found in the bone marrow.

32D cells are a hematopoietic progenitor cell line (144) often used to mimic HSPCs for *in vitro* assays. 32D cells can stay undifferentiated indefinitely as long as they are cultured with an IL-3 containing medium (121), where upon IL-3 removal from the media and introduction of G-CSF, they terminally differentiate to become granulocytes. 32D cells behaved very differently from HSPCs when they were cultured on the same set of collagen hydrogels and PA substrates.
Overall, they were approximately twice as large as HSPCs after 24 h of culture and showed a much more highly amorphous morphology ($CSI < 0.4$) on all substrates with a stiffness greater than 0.71 kPa (Fig. 2.7). Similar to primary HSPCs, their spread area tended to increase with an increasing substrate stiffness (Fig. 2.7(A)), but they showed very different biophysical response to varying ligand concentrations, with maximal spreading (largest size, lowest $CSI$) observed at intermediate (40 μg/mL) collagen densities (Fig. 2.8). Further, as a cell line their viability was not affected by decreasing collagen ligand density concentration. Overall, these results suggest that the use of 32D cells as a proxy of primary HSPCs may not be appropriate for experiments aiming to design artificial bone marrow mimics that present matrix-based cues.

Engler et al. showed that MSCs cultured on stiffer substrates (osteogenic PA substrates (34 kPa) and glass slides) were bigger in size with more substantial formation of stress fibers from cytoskeletal reorganization (27). More recently, Rowlands et al. performed an extensive study to decouple the effects of substrate stiffness, ligand presentation, ligand density on MSCs cultured on PA substrates with a wider range of stiffness and found similar trends in MSC spreading and stress fiber organizations (116). Interestingly, Chowdhury et al. found that ESC spreading was optimal at softer PA substrates while more differentiated cells derived from the same ESCs spread more with increasing substrate stiffness (84). These results clearly demonstrate that different types of cells behave differently when cultured on the same substrates, and that these differences may correlate with the degree of multipotency of the cells that were studied.

The use of a homologous series of two-dimensional PA substrates allowed effective analysis of the specific influence of substrate elasticity. However, such environments do not fully reconstitute the in vivo bone marrow microenvironments where cells are presented with 3D...
ECM-based cues. To probe the effect of dimensionality on cells, HSPC were cultured on top of or within soft collagen hydrogels. This system allowed HSPCs to be exposed to the identical substrate stiffness and ligand densities, with the only change coming in the dimensionality of how these extrinsic cues were presented. Due to the nature of the collagen hydrogels, however, this system was limited to low modulus settings (< 0.5 kPa) best representative of the soft microenvironment experienced by HSPCs within adipose tissue components of the bone marrow (23, 145). While subtle, the effect of construct dimensionality can be observed from the images of HSPCs (Fig. 2.3(B)) and 32D cells (Fig. 2.9). Qualitatively, HSPCs retained more rounded morphology in 3D collagen constructs while 32D cells developed large cytoplasmic protrusions that were larger in the stiffer constructs. While suggestive that primary HSPCs can rapidly respond to biophysical cues presented by their local microenvironment, significant future work will be required to fully unravel the complex relationship between the diverse microenvironments presented within the native bone marrow environment and the cell fate decisions made by individual HSPCs exposed to these environments. In addition to exploring HSPC biophysical responses to a wider range of substrate chemistries (i.e., ligand type, density) and mechanical properties, HSPCs provide the capacity to assess changes in stem cell functional properties through the use of in vitro colony forming unit assays (146) and in vivo bone marrow repopulation assays (147). Ongoing work is identifying changes not only in HSPC morphology but also in HSPC self-renewal versus myeloid/lymphoid differentiation via colony forming unit assays in response to substrate elasticity and ligand presentation.

2.6. Conclusions
This work describes the evaluation of the role biomaterial elasticity and ligand presentation play on the morphology and biophysical properties of primary murine HSPCs. Here we showed that HSPCs are responsive to biomaterial microenvironments over a range of substrate stiffness (Adipose tissue and bone marrow: <1 kPa; Cell-cell interactions: ~3 kPa; Bone-osteoid: ~100 kPa). Analysis of HSPC viability and subsequent changes in size and shape over a 24-hour culture period showed that HSPC spreading (increase in cell size, decrease in cell circularity) increased with substrate stiffness, but that effect was strongly influenced by receptor-ligand interactions. Notably, reducing collagen ligand density led to reduced HSPC spreading and viability. Importantly, we observed the biophysical response of 32D cells, a myeloid progenitor cell line used as a HSPC mimic, to be significantly different than that observed for primary HSPCs, suggesting that 32D cells may be a poor choice for experiments optimizing biomaterial constructs for HSPC culture. Most critically, because it was performed using only primary HSPCs and not a whole bone marrow population, this work confirms that matrix biophysical properties have the potential to directly impact HSPC fate. This work therefore suggests that biomaterial elasticity and ligand presentation may be critical design parameters for the development of artificial HSPC.
2.7. Figures

Figure 2.1. HSPC isolation scheme.
HSPCs were isolated from the femurs and tibias of 4 – 10-week-old C47Bl\6 mice via fluorescent-activated cell sorting (FACS) using conventional Lin$^-$/Sca-$^-$/c-Kit$^+$ gating strategies.
Figure 2.2. PA substrate characterization.
A) Stiffness of the PA substrates used in the experiments. PA substrate stiffness was characterized with AFM in our lab except for the 4% acrylamide/0.03% bis-acrylamide PA substrates whose stiffness was taken from reported values (124). B) Surface density of immobilized collagen on the PA substrate surface (Bound collagen) as a function of patterning solution concentration (Collagen conc.).
Figure 2.3. Effect of substrate dimensionality (2D vs. 3D) on HSPC viability and morphology.

A) Viability (%) of HSPCs cultured on top of 3D collagen hydrogels (2D) and within 3D collagen hydrogel constructs (3D) at 1.45 and 2.9 mg/mL. B) Images of HSPC cytoskeleton (F-actin) and nucleus (DAPI) gathered via confocal microscopy. Top two images show HSPCs cultured on top of collagen hydrogels (2D) and bottom two images show HSPCs cultured inside these same collagen constructs (3D). Yellow arrows: thin, filopodial protrusions enriched in F-actin fibers extending into the surrounding hydrogel. Scale bar: 5 µm.
Figure 2.4. Biophysical changes observed in HSPCs cultured on 2D substrates.
(collagen hydrogels – light gray bars; collagen-coated PA substrates – dark gray bars; glass substrates – blue bars) for 24 h. A) Highest HSPC viability was observed on lower stiffness substrates. *: $p < 0.05$. B) A significant effect of substrate stiffness was observed on HSPC spread area. HSPCs spread out significantly more on stiffest PA substrates (196 kPa) compared to softest PA substrates (0.71 kPa). *: $p < 0.05$ and **: $p < 0.001$ with respect to 0.71 kPa PA substrates. v: $p < 0.001$ with respect to glass (col+). C) Projected 2D images of HSPC cytoskeleton (F-actin) and nucleus (DAPI) gathered from confocal microscopy. Scale bar: 5 µm. D) Cell shape index (CSI) of HSPCs. *: $p < 0.05$. 
Figure 2.5. F-actin distribution in HSPCs cultured on PA substrates for 24 h.
A) Projected 2D images of HSPC cytoskeleton (F-actin) and nucleus (DAPI) gathered from confocal microscopy were analyzed by following the F-actin fluorescent intensity at three different arbitrarily-chosen cytoplasmic regions (denoted by three colored lines) from an arbitrary center to the cell edge. Scale bar: 5 µm. B) F-actin fluorescent intensity along one representative colored line. In order to avoid discrepancies arising from different laser settings, fluorescent intensity was normalized by quantifying the height (y) and width (x) of a peak observed near cell edge. Dashed black vertical line indicated determination of the cell edge. C) Normalized fluorescent intensity (y) of the cytoskeletal elements within the extranuclear cytosol increased with increasing substrate elasticity and then decreased substantially when HSPCs were cultured on bare glass coverslips, although the changes were subtle.
Figure 2.6. Effect of ligand density in HSPC spreading. 
A) Viability, B) spread area, and C) cell shape index (CSI) of HSPCs cultured on stiffest PA (196 kPa) substrate with varying ligand density (1 ng/mL, 40 μg/mL, 100 μg/mL). *: $p < 0.05$; **: $p < 0.001$. 
Figure 2.7. Biophysical changes observed in 32D cells cultured on 2D substrates. (collagen hydrogels – light gray bars; collagen-coated PA substrates – dark gray bars; glass substrates – blue bars) for 24 h. A) Within each subgroup, 32D cell spread area increased with increasing substrate stiffness of 3D collagen hydrogels and with that of 2D PA substrates. B) Projected 2D images of 32D cell cytoskeleton (F-actin) and nucleus (DAPI) gathered from confocal microscopy. 32D cells were overall larger than HSPCs, and spread out more with increasing substrate stiffness. Scale bar: 5 µm. C) Cell shape index (CSI) of 32D cells cultured on top of 3D collagen hydrogels or on top of 2D PA substrates. *: p < 0.05.
Figure 2.8. Effect of ligand density in the spreading of 32D cells.
A) Spread area and B) cell shape index (CSI) of 32D cells cultured on the stiffest PA substrate (196 kPa) with varying ligand density (1 ng/mL, 40 μg/mL, 100 μg/mL). *: p < 0.05.
Figure 2.9. Effect of substrate dimensionality (2D vs. 3D) on 32D cell morphology. Cells were fixed and stained to visualize cytoskeleton (F-actin; green) and nucleus (DAPI; blue). Top images show 32D cells cultured on top of collagen hydrogels (2D) and bottom two images show 32D cells cultured inside these collagen constructs (3D). Scale bar: 5 µm.
2.8. Tables

Table 2.1. Summary of the PA substrate stiffness.
Stiffness of PA gel substrates are summarized as reported in the literature (124) or experimentally characterized via AFM.

<table>
<thead>
<tr>
<th>% Acryl/% Bis-acrylamide (v/v%)</th>
<th>Young's Modulus (kPa)</th>
<th>Literature (124)</th>
<th>Experimentally Measured (uncoated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4/0.03</td>
<td></td>
<td>0.71 ± 0.24</td>
<td>---</td>
</tr>
<tr>
<td>5/0.10</td>
<td></td>
<td>3.15 ± 0.85</td>
<td>3.48 ± 0.59</td>
</tr>
<tr>
<td>5/0.26</td>
<td></td>
<td>8.58*</td>
<td>8.75 ± 1.21</td>
</tr>
<tr>
<td>38/2</td>
<td></td>
<td>---</td>
<td>196 ± 54.1</td>
</tr>
</tbody>
</table>

* Extrapolated from reported moduli
CHAPTER 3: Matrix Cues (Stiffness, Ligand Presentation) Rapidly Impact Fate Decisions of Hematopoietic Stem and Progenitor Cells

3.1. Abstract

Hematopoiesis is the physiological process where hematopoietic stem cells (HSCs) continuously generate the body’s complement of blood and immune cells within unique regions of the bone marrow. While imaging analyses have revealed gradients in cellular and extracellular matrix content across the marrow and while matrix elasticity and ligand presentation are believed strong regulators of stem cell fate, the role of the biophysical environment on HSC response is poorly understood. Here, we demonstrate using ECM protein-coated polyacrylamide (PA) substrates that the interplay between integrin activation and myosin-dependent processes significantly impacts the morphology, proliferation, and lineage specification of primary murine HSCs. Moreover, these changes appear linked to known microenvironmental transitions across the marrow. Combinations of matrix ligand (fibronectin) and matrix-associated stiffness were required to maintain hematopoietic progenitor populations, while matrix-ligand (laminin) signals were sufficient to enhance myeloid differentiation. Furthermore, inhibiting myosin II activation or α5β1 binding selectively abrogated the impact of the matrix environment on HSC fate. Notably, such fate decisions occurred within 3-hrs of HSCs being in culture.

3.2. Introduction

Hematopoietic stem cells are responsible for life-long hematopoiesis, the production of the full complement of blood and immune cells (3, 5, 6). Processes associated with HSC fate

---

2 This chapter has been adapted from the following manuscript in preparation: Choi JS & Harley BAC, Matrix cues rapidly impact early fate decisions of hematopoietic stem and progenitor cells. *In preparation.*
decisions occur within distinct subregions of the marrow, termed *niches* (1, 3, 8, 131, 148, 149). Notably, matrix biophysical cues (*e.g.*, mechanical properties, matrix-associated ligands) (25, 26, 34, 150, 151), biomolecular signals (*e.g.*, hypoxia, CXCL12) (148, 151-161), and local cell populations (*e.g.*, osteoblasts, mesenchymal stem cells (MSC), differentiated hematopoietic progeny) (50, 114, 162-165) vary significantly across the marrow. Further, HSCs exhibit dynamic behavior within the marrow, with quiescent HSCs remaining engrafted in the marrow and subfractions of activated HSCs mobilizing from and homing to the marrow via the peripheral blood. Hematopoietic homeostasis is hence believed to require a balance between a small number of *quiescent niches* and more prevalent *active niches*. Additionally, hematopoietic progenitor cells at discrete stages of differentiation have been observed to localize non-uniformly within the marrow; for example, Nombela-Arrieta et al. recently demonstrated using an image cytometry approach able to map the distributions of \(>10^5\) cells within the marrow that higher fractions of c-Kit\(^+\) hematopoietic progenitor cells associate with the endosteal region than the central marrow (25). The dynamic interplay between HSCs and a range of subregions of the bone marrow suggest that microenvironmental signals may be key regulators of HSC fate decisions such as *quiescence, self-renewal, and myeloid vs. lymphoid lineage specification* (24, 34).

Indeed, over the past two decades the subject of identifying the HSC niche has inspired a multitude of investigations (2). While the identity and composition of discrete niches remains unclear, putative HSCs have been identified near the bone endosteum (*endosteal niche*), in the central marrow cavity (*central medullary niche*) or the sinusoidal endothelium (*perivascular niche*) (1, 2, 4, 8, 9). Recent data suggest the *endosteal niche*, marked by a rich population of premature osteoblastic cells, houses quiescent, long-term repopulating HSCs and may mediate long-term maintenance of the HSC pool (1, 2, 4, 8, 35-37, 39, 40, 53). Found more deeply within
the marrow, the sinusoidal endothelium (*perivascular* niche) has been implicated in housing more active, self-renewing short term repopulating HSCs (ST-HSCs) (1, 2, 4, 8, 25, 29, 31, 44-46, 48-52). Very recently, an *arteriolar* niche associated with arteriole structures near the bone surface has also been suggested to contribute significantly to HSC quiescence (46). These subtle localization differences support the contention that quiescent and active niches exist in close spatial register across the marrow. However, teasing apart the complex constellations of signals that define cell fate-determining niches remains a challenge. While soluble biomolecules and putative niche cells have been correlated with HSC localization within the marrow, and despite the known structural, mechanical, and biomolecular diversity of the marrow itself, there exists no biophysical explanation for the niche-mediated regulation of HSC proliferation and lineage specification. The rarity of long term repopulating HSCs (LT-HSCs) within the marrow (<1:50,000 marrow cells) (3), and the difficulty of *in situ* imaging (57) has largely precluded more detailed study of the role matrix biophysical signals play in early HSC fate decisions.

Previous *in vitro* studies with primary hematopoietic cells that include varying subsets of hematopoietic stem and progenitor cells suggest the importance of matrix mechanics (93-95), topography (96-98), and paracrine signals (86) on the regulation of their fate decisions.

Here, we provide evidence of a direct link between the biophysical environment and early fate decisions using marrow-inspired ECM protein-coated polyacrylamide substrates as an engineered matrix platform with defined stiffness and ligand presentation. The design of the platform was motivated by the observation that the extracellular content of the bone marrow is spatially graded. Notably, near the bone surface is defined by stiffer microenvironments composing of osteoids and collagenous bone (~40 kPa) with higher fibronectin content while the microenvironments near the marrow vasculature is noted with higher expression of laminin and
the soft marrow that is rich in adipocytes (< 3 kPa) (23, 26). The use of mechanically tunable substrates permitted the examination of individual HSCs in response to defined matrix cues in vitro. Primary HSCs cultured on top of these substrates responded to changes in substrate stiffness and ligand presentation, where combinations of either or both of these factors impacted HSC fate decisions regarding proliferation vs. lineage specification towards myeloid lineages as indicated by colony-forming unit (CFU) assay results. Interestingly, substrates resembling the endosteal region (44 kPa; Fibronectin-coated) promoted proliferation of primitive myeloid progenitors while those resembling the vascular region (3.7 kPa; Laminin-coated) favored differentiation towards erythroid lineages, in line with the current niche models that suggest the endosteal niche hosts LT-HSC and supports HSC quiescence while the perivascular niche houses ST-HSCs and activated HSCs. Also, these fate decisions occurred rapidly, within 3-hours of culture HSCs in vitro.

3.3. Materials and Methods

3.3.1. Substrate preparation

PA gels were prepared in a 14-mm glass bottom culture dish (In Vitro Scientific, Sunnyvale, CA) as previously reported (94, 122, 124). In brief, a stock solution of 19:1 acrylamide and N,N’-methylenebisacrylamide was diluted in deionized water and an appropriate amount of 30% acrylamide solution was added to obtain desired concentrations of acrylamide and bis-acrylamide. Tetramethylethylenediamine (1/2,000 v/v) and 10% ammonium persulfate (1/200 v/v) were added to the mixture to initiate crosslinking via free radical formation. This solution was promptly placed onto the glass portion of the glass bottom dish pre-treated with 3-aminopropyltrimethoxysilane and 0.5% glutaraldehyde. A 12-mm glass coverslip treated with
dichlorodimethylsilane was placed on top of the solution to induce the formation of a thin gel with an even surface. Once polymerization was complete, the coverslip was carefully removed and the gel was washed twice with deionized water and stored at 4 °C until needed.

PA gel surface was functionalized with type I collagen, fibronectin, or laminin via photocrosslinking with Sulfo-SANPAH (94, 115). PA gel surface was first covered with 50 mM Sulfo-SANPAH and was exposed to a 365-nm UV source (UVP, Upland, CA) for 6 minutes. Upon rinsing the gel twice with 50 mM HEPES (pH 8.5), the procedure was repeated once more with fresh 50 mM Sulfo-SANPAH. Protein solution of type I collagen, fibronectin, or laminin (100 µg/mL) was placed on top and was left to react overnight at 4 °C. As previously reported (94), this procedure produced PA gels evenly coated with the protein (Fig. 3.1). Coated PA gels were used within two days of coating and sterilized with UV before use. All proteins were purchased from BD Biosciences, Bedford, MA. All other reagents were purchased from Fisher Scientific, Hampton, NH.

Coated glass substrates were prepared first by cleaning glass bottom dishes with 0.1 M HCl and rinsing thoroughly with sterile PBS and sterile deionized water. Protein solution (100 µg/mL) was then placed on top and incubated for 1 hour at 37 °C. Coated glass was used immediately.

For antibody blocking experiments, substrates were incubated with anti-α5β1 (Millipore, Billerica, MA) for 1 hour at room temperature before seeding cells. Manufacturer recommended dose was used.

For RGDfK-coated glass, glass bottom 96-well plates were used (In Vitro Scientific, Sunnyvale, CA). In brief, glass bottom well plates were cleaned and rendered hydrophilic by exposing to oxygen plasma for 5 min. The glass surface was then aminosilanized and annealed at
80 °C for 4 hours. The glass surface was subsequently reacted with mPEG-SVA and biotin-mPEG-SVA (10:1 ratio; MW 5,000; Laysan Bio, Arab, AL) and incubated with NeutrAvidin (Fisher Scientific, Hampton, NH) and biotin-RGDFK (Peptides International, Louisville, KY) to immobilize RGDFK on the surface (166).

3.3.2. HSC isolation

C57BL6 mice (The Jackson Laboratory, Bar Harbor, ME) between 4 and 10 weeks of age were euthanized with carbon dioxide in compliance with the University of Illinois Institutional Animal Care and Use Committee (IACUC) guidelines. Their femurs and tibias were gathered, crushed, and filtered through a 40-μm cell strainer (BD Falcon, Franklin Lakes, NJ) in PBS supplemented with 2% FBS (buffer) to collect whole bone marrow (WBM) cells. Red blood cells were removed by ACK lysis buffer. To prevent nonspecific binding, WBM cells were incubated with Fc receptor blocking antibody (CD16/CD32). They were then incubated with a cocktail of FITC-conjugated lineage (Lin) antibodies (CD5, CD45R (B220), CD11b, Anti-Gr-1 (Ly-6G/C), 7-4, Ter-119), PE-conjugated Sca-1, and APC-conjugated c-Kit. Propidium iodide was used to exclude dead cells. Labeled cells were sorted with FACS (BD FACS Aria™ cell sorter) to isolate Lin^−Sca-1^−c-Kit^+ (LSK) population (Fig. 3.2). This consistently yielded 4E4 – 6E4 LSKs per mouse. All antibodies were purchased from eBiosciences, San Diego, CA. All other reagents were purchased from Life Technologies, Grand Island, NY unless otherwise noted.

3.3.3. Cell culture

Isolated LSK cells were seeded on top of coated substrates at a seeding density of ~4,000 cells per sample. The low seeding density ensured minimal cell-cell interactions. StemPro®-34
serum free medium (Life Technologies, Grand Island, NY) supplemented with the complementary StemPro®-Nutrient Supplement, 100 ng/mL SCF, 100 ng/mL Flt3L, and 100 ng/mL TPO (Peprotech, Rocky Hill, NY) was used as the culture media. For myosin II inhibition studies, 100 µM blebbistatin (Sigma, St. Louis, MO) was added to the cell culture media at the start of culture.

3.3.4. Flow cytometric analysis

All flow cytometric analyses were performed with BD Biosciences LSR II flow cytometry analyzer. To assess changes in cell surface antigen expression, cultured HSCs were incubated with antibodies and analyzed to determine the percentage of Lin- and LSK subpopulations. For proliferation analysis, cells were stained with CellTrace™ Violet (Life Technologies, Grand Island, NY) according to the manufacturer’s protocol. For each cell division that the parental cell goes through, CellTrace™ Violet is distributed equally to its daughter cells. For the analysis of colony-forming cells from the CFU assay, colonies were harvested in bulk from the methylcellulose media after colony enumeration as previously reported (167). Collected cells were incubated with CD11b (Mac-1), Gr-1(Ly-6G/C), or a cocktail of LSK antibodies and analyzed for the fractions of cell populations expressing the corresponding antigen(s).

3.3.5. Colony-forming unit (CFU) assay

Cultured HSCs were lifted with TrypLE and resuspended in methylcellulose medium supplemented with murine cytokines (MethoCult® GF M3434; Stem Cell Technologies, Vancouver, Canada) according to the manufacturer’s protocol. In brief, the methylcellulose
medium containing the cells was dispensed to 35mm culture dishes using a 3cc syringe. Samples were prepared in triplicates or quadruplets and incubated for 11-14 days at 37° C and 5% CO₂ at which point the colonies that arose (CFU-GEMM, CFU-GM, CFU-M, CFU-G, CFU/BFU-E, CFU-Mk; Fig. 3.3) with more than 30 cells were enumerated with a light microscope (DMI4000, Leica Microsystems, Germany).

3.3.6. Statistical analysis

One-way analysis of variance (ANOVA) with Tukey post-hoc testing was used to determine statistical differences between data groups at a significance level of \( p < 0.05 \). All errors are reported as standard error of the mean (SEM) unless otherwise noted.

3.4. Results

To this end, we fabricated ECM protein-coated polyacrylamide (PA) gels whose biophysical properties were tuned to simulate stiffness (3.7, 44 kPa; Table 3.1) and ECM ligand presentation (type I collagen, fibronectin, laminin) of discrete bone marrow microenvironments (94, 124). We then isolated primary Lin⁻Sca-1⁺cKit⁺ (LSK) HSCs from marrow taken from femurs and tibias of C57BL6 mice via fluorescence-activated cell sorting (FACS; Fig. 3.2). We cultured isolated HSCs on ECM protein-coated PA gels or glass substrates for 24h to examine the effect of biophysical matrix cues on early HSC fate decisions.
3.4.1. **Biophysical changes in HSCs occur within 24h of culture and are linked to matrix environment**

After 24h in culture, flow cytometry analysis revealed the majority of HSCs stayed lineage negative (> 93%), but approximately 50% remained LSK (Fig. 3.4a, b), suggesting significant functional changes occurred. Matrix environment impacted HSC proliferation, with significant (p < 0.05) increases in HSC proliferative index on collagen versus fibronectin regardless of substrate stiffness (Fig. 3.5). Roughly 50% of the cells remaining attached to the substrate were still viable by the end of the 24h culture, regardless of substrate stiffness or ECM protein presented (Fig. 3.6a).

Phase contrast imaging (Fig. 3.6b) showed morphological changes in cultured HSCs as a function of matrix environment in line with our previous findings (94). Briefly, HSC appeared less circular and more irregular on stiffer substrates: spread area increased and their cell shape index (CSI; indicator of cell morphology), decreased with increasing substrate stiffness, (Fig. 3.4c, d). HSCs cultured on collagen-coated PA gels tended to spread more compared to those cultured on fibronectin- or laminin-coated PA gels particularly on softer gels (Fig. 3.4c) while HSCs appeared more spread with increasing substrate stiffness. These results indicate that matrix stiffness and ligand presentation induce significant changes in the HSC biophysical properties as early as 24h of culture.

3.4.2. **Functional changes in HSCs after initial matrix contact**

Analysis of HSC lineage commitment via colony-forming unit (CFU) assays showed lineage commitment of cultured HSCs was significantly altered as early as within 3 hours of *ex vivo* culture (Fig. 3.7). Within 3 hours of *ex vivo* culture on RGDfK-coated glass, HSCs were
activated and they significantly lost their multi-lineage repopulating potential, as indicated by significantly reduced numbers of CFU-GEMM colonies generated compared to freshly isolated HSCs \( (p < 0.05; \text{Fig. 3.7a}) \). Interestingly, engaging with RGDfK seemed to facilitate this process as the GEMM colony-forming potential of HSCs cultured on bareglass remained significantly greater than those cultured on RGDfK \( (p < 0.05) \), although still lower than that of freshly isolated HSCs. Additionally, HSCs cultured on RGDfK-coated glass showed rapid changes in lineage commitment towards myeloid and erythroid lineages (Fig. 3.7b, c). Bareglass cultures similarly supported commitment towards myeloid lineage but not towards erythroid lineage, suggesting that interactions with specific binding domains (in this case RGDfK) that activate selective integrins (in this case \( \alpha v \beta 3 \)) and related pathways have a differing role on HSC fate decisions particularly regarding proliferation vs. specific lineage specification. Once lineage specification decisions were made, these changes persisted as HSCs continued to be cultured for 24 hours (Fig. 3.7) \( (p < 0.05) \).

To get a comprehensive understanding of the role matrix cues (matrix stiffness, ligand presentation) play on HSC fate decisions, we cultured HSCs on ECM protein-coated PA gels with varying stiffness and coated glass then performed CFU assay to compare colony-forming patterns with those of freshly isolated HSCs (Fig. 3.8, 3.9). Discrete stages of myeloid specification appeared differentially impacted by matrix stiffness, matrix ligand presentation, or combinations thereof. Here, significantly fewer CFU-GEMM colonies, indicative of the presence of myeloid progenitors with multi-lineage differentiation potential, formed from HSCs cultured on laminin-coated PA gels independent of stiffness (3.7, 44 kPa; Fig. 3.8b). While matrix engagement did not impact CFU-GM colony (lineage-restricted myeloid progenitors) formation (Fig. 3.9a), matrix engagement differentially impacted subsequent CFU-G
(granulocyte) vs. CFU-M (macrophage/monocyte) specification. Notably, matrix engagement ubiquitously reduced CFU-G formation (Fig. 3.8e), while significantly more CFU-M colonies formed for HSCs cultured on fibronectin- or laminin-coated gels (Fig. 3.8c). Similarly, progenitors for erythrocytes (CFU-E/BFU-E) and megakaryocytes/platelets (CFU-Mk) showed divergent response, with significant increases in erythrocyte progenitor colonies found on laminin substrates while no ligand-dependent effect was observed on CFU-Mk colonies (Fig. 3.8d, f). These trends in the colony-forming potential of cultured vs. freshly isolated HSCs suggest that substrate stiffness and ECM ligand presentation can have individual or combinatorial effects on multiple stages of HSC lineage commitment.

3.4.3. Functional changes in HSCs as a function of matrix stiffness and ligand presentation

Substrate stiffness-dependent trends in colony formation were observed in CFU-GEMM and CFU/BFU-E colonies (Fig. 3.8). CFU-GEMM colonies formation increased significantly on fibronectin-coated 44 kPa versus 3.7 kPa PA gels (Fig. 3.8b). Similarly, CFU/BFU-E colony formation increased significantly on laminin-coated 44 kPa versus 3.7 kPa PA gels (Fig. 3.8f). These results suggest that fibronectin promotes the maintenance of more primitive hematopoietic progenitors while laminin favors commitment towards erythroid lineages, particularly at surface stiffness associated with extracellular matrix interactions (44 kPa).

When we also accounted for HSCs cultures on specific ligand-coated glass in addition to ligand-coated PA gels, matrix-mediated effects particularly regarding changes in matrix stiffness often appeared to be enhanced. Overall, while there were few differences in total numbers of formed colonies between ECM groups (Table 3.2; Fig. 3.9b), significant differences were observed in the type of colonies formed (Fig. 3.8, 3.9). CFU-M colonies formed significantly
more with increasing stiffness on collagen- or fibronectin-coated substrates \((p < 0.05; \text{Fig. 3.8c})\). Similarly, CFU-Mk colonies formed significantly more on fibronectin-coated substrates with increasing stiffness \((p < 0.05; \text{Fig. 3.8d})\). CFU/BFU-E colony numbers also rose with increasing stiffness on collagen- or laminin-coated substrates \((p < 0.05; \text{Fig. 3.8f})\). Meanwhile, HSCs cultured on coated-glass produced highest numbers of CFU-M and CFU/BFU-E colonies regardless of the ECM protein presented, suggesting that culturing HSCs on stiff substrates which include most conventional in vitro culture substrates may be more important than ECM ligand presentation for commitment towards monocytic and erythroid lineages.

Results of CFU assays were also considered in light of known differences in matrix ligand and mechanical properties across the native marrow. Notably, fibronectin-coated substrates promoted the maintenance of primitive myeloid progenitors when the stiffness was 44 kPa or higher and to monocytic and megakaryocyte lineages at all stiffness (Table 3.2; Fig. 3.8b, c, d). Laminin-coated substrates supported later stage, lineage-restricted myeloid progenitors, notably to megakaryocyte lineage at 3.7 kPa and to monocytic and erythroid lineages at 44 kPa (Table 3.2; Fig. 3.8e, d, f). Further, while type I collagen enhanced proliferative index (Fig. 3.5), it seemed to play a minor role in delineating HSCs commitment towards myeloid lineages compared to fibronectin or laminin (Table 3.2).

### 3.4.4. Actomyosin contractility impacts early HSC fate

To assess the role of actomyosin contractility in the early phase regulation of HSC fate, we cultured primary HSCs on top of the ECM-coated PA gels and glass substrates with blebbistatin, a known inhibitor of myosin II, for 24h. Blebbistatin did not significantly affect cell spreading or morphology during this time frame although cells spread out slightly less and
appeared a little more circular with blebbistatin (Fig. 3.10). With myosin II function largely blocked by blebbistatin, previously observed substrate stiffness-dependent trends in colony formations of cultured HSCs were no longer present ($p < 0.05$; Fig. 3.11, 3.12). Moreover, blebbistatin significantly altered the colony-forming potential of most colonies. CFU-GEMM colonies formed significantly less on fibronectin- or laminin-coated substrates ($p < 0.05$) while similar numbers formed on collagen-coated substrates (Fig. 3.11a). Flow cytometric analysis of the colony-forming cells from HSCs cultured on fibronectin-coated substrates also showed decreased numbers of LSK cells with blebbistatin, which agreed with these findings (Fig. 3.13). In contrast, CFU-G colony formation significantly increased regardless of substrate stiffness or ECM protein presented when blebbistatin was added to HSC cultures ($p < 0.05$; Fig. 3.11b).

Interestingly, ECM ligand presentation played an important role even when myosin II-mediated contraction was inhibited to reduce intracellular tension, leading to different colony numbers particularly between laminin and the other proteins (fibronectin, type I collagen) (Fig. 3.11, 3.12). This trend was observed in all colonies except for CFU-GEMM and CFU-G colonies mentioned above. Notably, fibronectin and collagen coating favored decreased formations of most of these colonies while laminin coating supported increased formations. For instance, CFU/BFU-E colonies formed significantly less when HSCs were cultured on collagen- or fibronectin-coated substrates ($p < 0.05$) but not on laminin-coated substrates, with the exception of 44 kPa ($p < 0.05$; Fig. 3.11c). Similarly, CFU-M colonies formed significantly less when HSCs were cultured on collagen- or fibronectin-coated substrates but instead significantly jumped on laminin-coated substrates ($p < 0.05$; Fig. 3.12b). For CFU-GM and CFU-Mk colonies, colony numbers stayed similar or decreased when HSCs were cultured on collagen- or
fibronectin-coated substrates but significantly jumped on laminin-coated substrates \((p < 0.05; \text{Fig. 3.12a; c})\).

Another interesting point was that with actomyosin contractility blocked with blebbistatin, colony-forming potential of HSCs cultured on collagen- or fibronectin-coated substrates did not show any statistical differences with respect to that of freshly isolated HSCs (solid blue lines), with the only exception of CFU-G colonies from HSCs on 3.7 kPa collagen-coated PA gels (Fig. 3.11, 3.12). In contrast, colony-forming potential of HSCs cultured on laminin-coated substrates significantly deviated from that of freshly isolated HSCs in all colony types. CFU-GEMM colony numbers significantly decreased (Fig. 3.11a) while CFU-G (Fig. 3.11b), CFU/BFU-E (Fig. 3.11c), CFU-GM (Fig. 3.12a), CFU-M (Fig. 3.12b), and CFU-Mk (Fig. 3.12c) colony numbers significant increased \((p < 0.05)\), leading to an increase in the total number of colonies (Fig. 3.12d).

3.4.5. Integrin activation on the regulation of early HSC fate

To assess the role of integrin activation on the regulation of HSC fate decisions, we pre-treated fibronectin-coated PA gels with α5β1 antibody to block binding via RGD binding domain then cultured primary HSCs on top for 24h. When CFU assay was performed, this resulted in a significant decrease in CFU-GEMM (Fig. 3.14a) and CFU-M (Fig. 3.14b) colonies and a significant increase in CFU-G (Fig. 3.14c) and CFU-GM (Fig. 3.15a) colonies \((p < 0.05)\). In contrast, CFU/BFU-E (Fig. 3.14d) and CFU-Mk (Fig. 3.15b) colonies were unaffected. For the most part, these results are consistent with earlier observations made when blebbistatin was added, with the exception of CFU/BFU-E and CFU-GM colonies. In particular, CFU/BFU-E colonies formed less when myosin II was inhibited but stayed unaffected in this case. Moreover,
3.5. Discussion

Using marrow-inspired ECM protein-coated PA gel and glass substrates, we obtained evidence of a direct link between the biophysical environment and HSC fate decisions particularly regarding proliferation vs. lineage specification. We found that such fate decisions occur rapidly, as early as within 3-hours of ex vivo cultures. Also, marrow-inspired matrix cues had complex, combinatorial effects on HSC fate decisions, where combinations of factors (e.g., substrate stiffness and ligand presentation) at specific density (e.g., 3.7 vs. 44 kPa or Fn vs. Ln vs. Col I) had differential impact on HSC fate decisions. While these matrix cues also induced varying degrees of changes in cell spreading and morphology of HSCs, these differences were subtle and overall HSCs spread more while appearing less circular with increasing substrate stiffness. Overall proliferation activity was higher when HSCs were cultured on collagen-compared to fibronectin-coated PA gels, suggesting that specific ligands may support the activation of HSCs or the selective proliferation of activated HSCs. Due to the lack of suitable functional metrics that could continuously monitor HSC activity in vitro, in situ, it is difficult to know if our observations are a direct result of presented cues inducing endogenous changes or if the presented cues simply selectively expand subsets of the cultured cell population. However, our CFU assay results suggest that both the former (i.e., quiescence/self-renewal vs. lineage commitment) and the latter (i.e., lineage specification) depend on specific matrix cues.

Notably, fibronectin-coated substrates with a stiffness of 44 kPa or higher supported primitive hematopoietic stem and progenitor cells (i.e, increased numbers of CFU-GEMM colony numbers dropped even more in this case ($p < 0.001$), suggesting that integrin activation is critical in commitment towards these lineages.
colonies) while laminin-coated substrates favored commitment towards erythroid lineage (i.e., increased numbers of CFU/BFU-E colonies). Interestingly, these conditions closely resemble the microenvironments of the endosteal (high fibronectin content, stiffness of ~40 kPa or higher) and perivascular (high laminin content) regions, respectively, providing evidence that microenvironmental context-driven biophysical cues have a differing role in the regulation of HSC fate decisions.

In the bone marrow, HSCs and progenitors are exposed to a number of biophysical matrix cues and mechanical forces in their microenvironments, including elasticity, nanotopography, spatial distribution of adhesive motifs and ligands, pressure, and stretching (24). Evidence suggests that extracellular biophysical cues cause changes in the mechanotransduction of HSCs, especially in intracellular tension, which leads to altered HSC fate (24, 34). Changes in intracellular tension are mediated by myosin, which dynamically crosslinks and pulls on actin filaments (168). Hematopoietic cells are known to express A and B isoforms of mammalian non-muscle myosin II (‘myosin II’) (34) and while its role in the dynamic regulation of HSC fate decisions remains unclear, previous reports implicate that myosin II function is critical in several lymphoid and myeloid lineages including T cell motility (169), B cell receptor-driven antigen presentation (170), neutrophil chemotaxis (171), macrophage phagocytosis (172), and enucleation in erythroid lineages in suspension cultures (173). Also, granulocytes were found to be highly deformable than their progenitors, which likely facilitates their migration across the endothelial barrier (174). Indeed, inhibiting myosin II-mediated contraction using blebbistatin significantly increased commitment towards granulocytic lineage (i.e., increase in CFU-G colony numbers). Disrupting intracellular tension also altered commitment towards other lineages. Notably, while this shifted the colony-forming potential of HSCs cultured on collagen- or
fibronectin-coated substrates to the levels of freshly isolated HSCs, laminin-coated substrates significantly expanded lineage-restricted downstream progenitors, as indicated by a significant increase in the numbers of CFU-GM, -M, -G, -E, and -Mk colonies. These results suggest that matrix engagement via specific ligands prior to actomyosin contraction critically impacts HSC fate decisions by triggering selective integrin-mediated signaling pathways.

It is believed that a cell first attaches to a substrate when its integrins engage ECM ligands, which recruit talin and kindlin to the cytoplasmic end of the integrins to strengthen the bond (175, 176). This is followed by integrin clustering and generation of adhesion complexes. A typical adhesion complex formation progresses through the formation of nascent adhesions (submicron-size) followed by the formation of focal complexes (~1 μm), focal adhesions (typically ~1 μm and 3-5 μm long), and fibrillar adhesions (located near cell centers, mostly in fibroblasts) as the adhesion site matures (175). Unlike many anchorage-dependent cells that form focal adhesions, HSCs do not form distinct focal adhesions although we previously observed active cytoskeletal reorganization within the first 24h of culture (94); though given the findings here, much of the cytoskeletal reorganization may be attributed to sub-fractions that have already begun lineage specification. When nascent adhesions form, several focal adhesion proteins are recruited and form a complex but it may be too small to be visualized (175). Future endeavors targeting improved understanding of HSC engagement with well-defined matrix substrates would contribute significant new information regarding the impact of matrix signals on HSC fate decisions. Also, integrin activation and clustering in nascent adhesions are independent of myosin II activity, although these still involve actin polymerization (177).

In line with this, our CFU assay results suggest that a series of signaling molecules and pathways involved in the early stage of adhesion (i.e., initial attachment, integrin activation, and
clustering) may be more closely linked to HSCs committing to a specific lineage than those involved in the later stages that require actomyosin contractility. When binding via RGD is blocked, fibronectin-coated PA gels could no longer support primitive hematopoietic stem and progenitors (i.e., a significant drop in the number of CFU-GEMM colonies, even more than when myosin II-mediated contraction was inhibited). Lineage specification patterns also changed, where commitment to monocytic lineage significantly decreased but commitment to granulocytic lineage significantly decreased. Lineage commitment to erythroid or megakaryocytic lineages remained unchanged. These observations implicate that specific integrin activation differentially impacts HSC fate decisions.

3.6. Conclusions

Here, we provide evidence of a direct link between the biophysical environment and early fate decisions using marrow-inspired ECM protein-coated polyacrylamide substrates as an engineered matrix platform with defined stiffness and ligand presentation. Specifically, our CFU data highlights the importance of integrin activation and actomyosin contraction in the dynamic regulation of early HSC fate, particularly regarding quiescence/self-renewal vs. lineage specification. Notably, microenvironmental conditions resembling the endosteal niche (high fibronectin content, stiffer) supported primitive hematopoietic stem and progenitor cells while those resembling the perivascular niche (high laminin content) encouraged differentiation activity, particularly towards erythroid lineage via myosin II-dependent contractility and α5β1-mediated adhesion. Taken together, these findings establish the utility and relevance for using context-driven matrix cues as design parameters for building an in vitro platform for the expansion or selective differentiation of HSCs in future applications.
3.7. Figures

Figure 3.1. ECM protein coating on polyacrylamide (PA) gels and glass. Collagen type I-functionalized PA gels and class were visualized via immunofluorescence.
Figure 3.2. FACS scheme to isolate LSK cells.
LSK (Lin⁻Sca-1⁺c-kit⁺) cell population was isolated from mouse femurs and tibias via FACS.
Figure 3.3. Colonies arising from CFU assay.
Colonies were enumerated after 11-14 days of incubation in methylcellulose media. CFU-GEMM, CFU-GM, CFU-M, CFU-G, CFU/BFU-E, and CFU-Mk were counted. Scale bar: 200 µm.
Figure 3.4. Changes in surface antigen expression and morphology after culturing HSCs for 24h.
Cultured HSCs were analyzed with flow cytometry to determine their surface antigen expression a, Lin- (%) and b, LSK (%) cell populations (n = 3). c, Spread area and d, CSI were quantified from phase contrast images (n = 24-45). Red line represents HSCs cultured on bareglass. Blue line represents the area of a freshly isolated HSC (diameter: ~6 µm). *: p<0.05. #: p<0.05 with respect to 3.7 kPa PA gel sample with the same ECM protein coating.
Figure 3.5. Proliferation activity of HSCs after 24h in culture.
Cultured HSCs were analyzed with flow cytometry to determine their proliferation activity (n = 3). *: $p<0.05$, **: $p<0.001$. 
Figure 3.6. Live fractions of attached cells and phase contrast images of cultured HSCs. 

**a,** % Live cells remaining attached to the substrates after 24h culture (n = 5-15). Red line represents the bareglass sample. **b,** Phase contrast images of cultured HSCs. Scale bar: 10 µm.
Figure 3.7. CFU assay results for HSCs cultured on c-RGD-coated glass. 
a, CFU-GEMM, b, CFU-M, and c, CFU-E colonies were enumerated for HSCs cultured on c-RGD coated glass. These results were compared freshly isolated HSCs (blue bars; \( p < 0.05 \) indicated by blue asterisk) and HSCs cultured on bareglass (striped bars; \( p < 0.05 \) indicated by black asterisk). As these results indicate, lineage specification is determined rapidly, as early as within 3-hours of \textit{ex vivo} culture.
Figure 3.8. CFU assay results for HSCs cultured for 24h.

a, CFU assay was performed with cultured HSCs to assess their lineage commitment states down the myeloid lineage. b, CFU-GEMM, c, CFU-M, d, CFU-Mk, e, CFU-G, f, CFU/BFU-E (n = 3-7) are shown. Blue line represents the number of colonies that arose from freshly isolated HSCs. *: p<0.05. *: p<0.05 and **: p<0.001 (blue) with respect to colonies from freshly isolated HSCs.
Figure 3.9. CFU assay results for HSCs cultured for 24h (continued).
CFU assay was performed with cultured HSCs to assess their lineage commitment states down the myeloid lineage. a, CFU-GM, b, Total number of CFU colonies (n = 3-7). Blue line represents the number of colonies that arose from freshly isolated HSCs. *: p<0.05. *: p<0.05 (blue) with respect to colonies from freshly isolated HSCs.
Figure 3.10. Cell spreading and morphology in HSCs cultured with blebbistatin.
HSCs were cultured with blebbistatin (100 µM; “BB”) added to the media. Their a, spread area and b, CSI are shown (n = 29-35).
Figure 3.11. CFU assay results for HSCs cultured with blebbistatin.
CFU assay was performed with HSCs cultured with blebbistatin added to the culture media (n = 3-7). a, CFU-GEMM, b, CFU-G and c, CFU/BFU-E. *: p<0.05. *: p<0.05 and **: p<0.001 (red) with respect to the same substrate condition without blebbistatin. *: p<0.05 and **: p<0.001 (blue) with respect to colonies from freshly isolated HSCs.
Figure 3.12. CFU assay results for HSCs cultured with blebbistatin (continued).
CFU assay was performed with HSCs cultured with blebbistatin added to the culture media (n = 3-7). a, CFU-GM, b, CFU-M and c, CFU-Mk, d, Total number of CFU colonies. *: p<0.05. *: p<0.05 and **: p<0.001 (red) with respect to the same substrate condition without blebbistatin. *: p<0.05 and **: p<0.001 (blue) with respect to colonies from freshly isolated HSCs.
Figure 3.13. Flow cytometric analysis of the colony-forming cells harvested from CFU assay.

After colony enumeration, colony-forming cells from CFU-GEMM, CFU-M, and CFU-G colonies were harvested from the methylcellulose media and stained for flow cytometric analysis. Populations that are a, LSK, b, Mac-1+, and c, Gr-1+ (n = 3) from CFU-GEMM, CFU-M, CFU-G colony-forming cells, respectively, are shown.
Figure 3.14. CFU assay results for HSCs cultured when binding via α5β1 is blocked. CFU assay was performed with HSCs cultured on fibronectin-coated PA gels pre-treated to block binding via α5β1 (n = 3-4). a, CFU-GEMM, b, CFU-M c, CFU-G, d, CFU/BFU-E colonies. *: p<0.05 and **: p<0.001 (orange) with respect to the same substrate condition without pre-treatment. **: p<0.001 (blue) with respect to colonies from freshly isolated HSCs.
Figure 3.15. CFU assay results for HSCs cultured when binding via α5β1 is blocked (continued).
CFU assay was performed with HSCs cultured on fibronectin-coated PA gels pre-treated to block binding via α5β1 (n = 3-4). a, CFU-GM, b, CFU-Mk, c, Total number of colonies. *: p<0.05 (orange) with respect to the same substrate condition without pre-treatment.
3.8. Tables

Table 3.1. Stiffness of polyacrylamide (PA) gels.
Stiffness of PA gels measured by AFM force-indentations are summarized here. For detailed experimental conditions and procedures, refer to §4.2 and (94).

<table>
<thead>
<tr>
<th>%Acrylamide/%Bis-acrylamide</th>
<th>Elastic Modulus, E (kPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5/0.1</td>
<td>3.7 ± 0.4</td>
</tr>
<tr>
<td>10/0.3</td>
<td>44 ± 16.1</td>
</tr>
</tbody>
</table>
Table 3.2. Statistical significance (p values) in different ECM coatings in producing CFU colonies.
This table summarizes the p values between substrates coated with different ECM proteins in order to assess which ECM protein is linked to an increase or a decrease in a particular CFU colony formation. *: p<0.05.

<table>
<thead>
<tr>
<th>Colony Type</th>
<th>Stiffness</th>
<th>3.7 kPa</th>
<th>44 kPa</th>
<th>Coated glass</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fn vs. Col: p = 0.38578</td>
<td>Fn vs. Col: p = 0.02524*</td>
<td>Fn vs. Col: p = 0.01704*</td>
</tr>
<tr>
<td>GEMM</td>
<td></td>
<td>Fn vs. Ln: p = 0.95544</td>
<td>Fn vs. Col: p = 0.62189</td>
<td>Ln vs. Ln: p = 0.95667</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Col vs. Ln: p = 0.26935</td>
<td>Col vs. Ln: p = 0.9626</td>
<td>Col vs. Ln: p = 0.07034</td>
</tr>
<tr>
<td>G</td>
<td>Fn vs. Col: p = 0.01749*</td>
<td>Fn vs. Col: p = 0.00189*</td>
<td>Fn vs. Col: p = 0.04276*</td>
<td>Fn vs. Col: p = 0.01516*</td>
</tr>
<tr>
<td></td>
<td>Fn vs. Ln: p = 0.51669</td>
<td>Fn vs. Ln: p = 0.00307*</td>
<td>Ln vs. Ln: p = 0.00307*</td>
<td>Ln vs. Ln: p = 0.66394</td>
</tr>
<tr>
<td></td>
<td>Col vs. Ln: p = 0.0697</td>
<td>Col vs. Ln: p = 0.00307*</td>
<td>Ln vs. Ln: p = 0.00307*</td>
<td>Ln vs. Ln: p = 0.66394</td>
</tr>
<tr>
<td>Mk</td>
<td>Fn vs. Col: p = 0.03533*</td>
<td>Fn vs. Col: p = 0.01167*</td>
<td>Fn vs. Col: p = 0.03928*</td>
<td>Fn vs. Col: p = 0.03928*</td>
</tr>
<tr>
<td></td>
<td>Fn vs. Ln: p = 0.95006</td>
<td>Fn vs. Ln: p = 0.09011</td>
<td>Ln vs. Ln: p = 0.00307*</td>
<td>Ln vs. Ln: p = 0.55448</td>
</tr>
<tr>
<td></td>
<td>Col vs. Ln: p = 0.03533*</td>
<td>Col vs. Ln: p = 0.26935</td>
<td>Ln vs. Ln: p = 0.00307*</td>
<td>Ln vs. Ln: p = 0.15408</td>
</tr>
<tr>
<td>G</td>
<td>Fn vs. Col: p = 0.23663</td>
<td>Fn vs. Col: p = 0.01659</td>
<td>Fn vs. Col: p = 0.1565</td>
<td>Fn vs. Col: p = 0.1565</td>
</tr>
<tr>
<td></td>
<td>Fn vs. Ln: p = 0.95006</td>
<td>Fn vs. Ln: p = 0.73099</td>
<td>Ln vs. Ln: p = 0.00307*</td>
<td>Ln vs. Ln: p = 0.15408</td>
</tr>
<tr>
<td></td>
<td>Col vs. Ln: p = 0.34283</td>
<td>Col vs. Ln: p = 0.73099</td>
<td>Ln vs. Ln: p = 0.00307*</td>
<td>Ln vs. Ln: p = 0.15408</td>
</tr>
<tr>
<td>E</td>
<td>Ln vs. Col: p = 0.12278</td>
<td>Ln vs. Col: p = 0.0054</td>
<td>Ln vs. Col: p = 0.80675</td>
<td>Ln vs. Col: p = 0.80675</td>
</tr>
<tr>
<td></td>
<td>Ln vs. Fn: p = 0.08567</td>
<td>Ln vs. Fn: p = 0.00292*</td>
<td>Ln vs. Ln: p = 0.57039</td>
<td>Ln vs. Ln: p = 0.57039</td>
</tr>
<tr>
<td></td>
<td>Col vs. Fn: p = 0.95723</td>
<td>Col vs. Fn: p = 0.79419</td>
<td>Ln vs. Ln: p = 0.9072</td>
<td>Ln vs. Ln: p = 0.9072</td>
</tr>
<tr>
<td>Total # of colonies</td>
<td>Fn vs. Col: p = 0.09708</td>
<td>Fn vs. Col: p = 0.01464*</td>
<td>Fn vs. Col: p = 0.12996</td>
<td>Fn vs. Col: p = 0.12996</td>
</tr>
<tr>
<td></td>
<td>Fn vs. Ln: p = 0.99526</td>
<td>Fn vs. Ln: p = 0.94659</td>
<td>Ln vs. Ln: p = 0.08284</td>
<td>Ln vs. Ln: p = 0.08284</td>
</tr>
<tr>
<td></td>
<td>Col vs. Ln: p = 0.10928</td>
<td>Col vs. Ln: p = 0.01041*</td>
<td>Ln vs. Ln: p = 0.93411</td>
<td>Ln vs. Ln: p = 0.93411</td>
</tr>
</tbody>
</table>
CHAPTER 4: SINGLE CELL ANALYSIS METHODS FOR QUANTIFYING FUNCTIONAL OUTPUT OF HEMATOPOIETIC STEM CELLS

4.1. General chapter overview

Current limitation to continuously monitor the functional changes in hematopoietic stem cells (HSCs) in vitro limits our understanding of how matrix signals inform HSC fate decisions (57, 58). Currently available functional assays of HSCs include in vivo repopulation as well as in vitro colony-forming unit (CFU), long-term culture-initiating cell (LTC-IC), and cobblestone area-forming cell (CAFC) assays (58, 59). All of these approaches require long incubation time and are expensive and labor-intensive. Further, they do not allow continuous, label-free analysis of putative HSCs in situ. Recent efforts by Schroeder et al. have highlighted the potential of using continuous, time-lapse imaging assays to trace HSC bioactivity (56, 57, 108, 178); however, as these require the use of fluorescent microscopy and combinations of multiple fluorescent labels such approaches are ultimately expensive and limited in the scope of their analyses. Means to dynamically monitor single putative HSCs in situ in a label-free manner would therefore supply technical platforms to better understand the nuances of HSC fate decision processes. To explore the feasibility of new single cell analysis methods as functional metrics of HSCs, we evaluated atomic force microscopy (AFM), time-of-flight secondary ion mass

---

3 This chapter has been adapted from the following publication and manuscripts in preparation:

Choi JS & Harley BAC, Atomic force microscopy indentation of soft samples for efficient extraction of the Young’s modulus from the AFM force curve data. In preparation.


Choi JS, Yelena Ilin, Mary Kraft, Harley BAC, Identifying differentiation stage and proliferation state of individual primary hematopoietic cells by multivariate analysis of Raman spectra. In preparation.
spectroscopy (TOF-SIMS) (179), and Raman spectroscopy approaches in their ability to resolve characteristic parameters of single hematopoietic stem and progenitor cells. Raman spectroscopy appears particularly attractive because it offers the potential for live imaging of single cells ex vivo. TOF-SIMS data revealed compositional differences in the cell membranes of primary hematopoietic cells but this method is limited to the analysis of fixed samples. AFM was not suitable for the analysis of hematopoietic stem and progenitor cells due to large lateral instability during force indentations although it was useful for the mechanical characterization of soft hydrogels.

From the outset I want to acknowledge the highly collaborative nature of a major element of this chapter. Notably, §4.3 (Time-of-Flight Secondary Ion Mass Spectroscopy) and §4.4 (Raman spectroscopy) would not be possible without the assistance and expertise of Prof. Mary Kraft and her laboratory at the University of Illinois at Urbana-Champaign. Section §4.3 is modified from a joint manuscript co-authored with Dr. Jessica Frisz and Dr. Robert Wilson (Kraft Lab). Section §4.4 is modified from a joint manuscript currently in preparation with Yelena Ilin (Kraft Lab). I chose to include the entire technical content of both manuscripts in this chapter as it adds significant perspective to this thesis, but from the outset want to acknowledge the contributions of my co-authors and collaborators in these projects.

4.2. Atomic Force Microscopy

4.2.1. Abstract

It is becoming increasingly apparent that continued progress in the field of tissue engineering requires appropriate tools that facilitate mechanical characterization of soft, hydrated samples ranging from live cells to biomaterials and native tissue specimens. Mechanical
characterization of such soft samples often uses atomic force microscopy (AFM) as it allows samples to be probed *in situ*. Notably, this capability enables spatial analysis of the sample in a label-free manner in experimentally patterned settings, even on live samples. To calculate the Young’s modulus of the sample, a Hertzian contact model is typically used to fit the AFM force indentation data. Application of the Hertz model requires an accurate and precise determination of the contact point or when the AFM tip first comes in contact with the sample. While several strategies have been developed to determine the contact point, these often introduce large errors or are too rigorous for the frequent force mapping of a large number of samples. Here, we describe a simple calculation scheme that could be easily employed to compute the Young’s modulus of soft samples, including soft hydrogels and biological samples. We also explored the effect of the assumptions made on several parameters during the analysis and performed compression testing to compare the Young’s moduli obtained. This approach may be useful for the large-scale analysis of AFM force data to characterize soft samples.

4.2.2. Introduction

Growing interest in tissue engineering applications and other bioengineering approaches requires the mechanical characterization of soft samples ranging from live cells to hydrogels and biomaterials. Recently, advances in characterization tools have enabled studies correlating changes in cellular mechanics with changes in cell stemness (84) or pathological state (*e.g.*, metastatic potential) (180, 181). Monitoring changes in cell stiffness may therefore serve as a marker for important changes to cellular states. Increasingly, soft biocompliable hydrogels such as polyacrylamide are widely used as 2D or 3D platforms to culture cells *in vitro* (94, 127, 182-185). As evidence increasingly points to context-driven matrix cues as important regulators of
cell fate decisions (27), the use of physiologically relevant cell culture platforms has become extensive, and the substrates increasingly complex.

Atomic force microscopy (AFM) is often used for the mechanical characterization of soft samples because it allows samples to be probed \textit{in situ}. This enables spatial resolution of mechanical analysis of specimens in a label-free manner. To calculate the Young’s modulus of the sample, Hertz model is typically used to fit the AFM force indentation data. The commonly used Hertzian contact model describes the contact mechanics between an elastic sphere and a rigid flat surface and assumes linear elastic behavior with negligible adhesion (186-190). Extensions of the Hertz model to account for various geometries and rigorous mathematical models to include surface forces have been developed (186, 191). Application of the Hertz model to assess mechanical properties of soft, hydrated biological substrates requires an accurate and precise determination of the contact point, where the AFM tip first comes in contact with the sample (186, 189). While the Hertz model provides a good estimate of the sample Young’s modulus for most cases, the assumptions made during the analysis may lead to large errors.

Several strategies have been developed to determine the contact point; however, these often introduce large errors or are too rigorous for the frequent force mapping of a large number of samples (189, 190, 192). In addition, several assumptions (e.g., Hertzian model neglects adhesive interactions between the tip and the sample or the presence of other surface forces) made during the calculations but they are seldom explicitly examined or reported, leading to discrepancies between the published values of the elastic properties of the same samples across different laboratories.

In this paper, we describe a simple calculation scheme to compute the Young’s modulus of soft samples, including soft hydrogels and biological samples. We also explored the effect of
the assumptions made on several parameters during the analysis and performed compression testing to compare the Young’s moduli obtained. Such an approach may be very useful for large-scale AFM force data analysis of soft samples.

4.2.3. Materials and Methods

4.2.3.1. Sample preparation for AFM

Polyacrylamide (PA) gels were prepared as previously reported (94, 122, 124). In brief, a stock solution of 19:1 acrylamide and N,N’-methylenebisacrylamide mixture was diluted with deionized water and an appropriate amount of 30% acrylamide solution was added to obtain a working solution with desired acrylamide and bis-acrylamide concentrations. The stiffness of the PA gels can be controlled by varying the concentrations of acrylamide and/or bis-acrylamide. Tetramethylethylenediamine (TEMED; 1/2,000 v/v) and 10% ammonium persulfate (1/200 v/v) were added to the solution to initiate crosslinking via free radical formation. This mixture was promptly placed on a glass slide previously treated with 3-aminopropyltrimethoxysilane and 0.5% glutaraldehyde. A 12-mm glass coverslip treated with dichlorodimethylsilane was placed on top of the mixture to induce the formation of a thin, flat gel. Polymerization was facilitated by incubating it at 37 °C for 30 minutes. The glass coverslip on top was then carefully removed and the gel was washed twice with deionized water and stored hydrated at 4 °C until needed. This resulted in a thin PA gel with a thickness of ~70 μm. PA gel variants with five different stiffnesses were created (%acrylamide/%bis-acrylamide: 5/0.10, 5/0.26, 10/0.3, 15/0.6, 38/2). All reagents were purchased from Fisher Scientific (Hampton, NH).
4.2.3.2. Sample preparation for compression testing

Acrylamide/bis-acrylamide stock solution diluted to desired concentrations was mixed with TEMED and 10% ammonium persulfate as described earlier. This solution was then placed between two glass slides separated by spacers and incubated at 37 °C for 30 minutes for complete polymerization. This resulted in a thin PA gel sheet with an average thickness of 1.055 ± 0.134 mm. Using a 8-mm biopsy punch, PA gel discs were punched out and kept hydrated in deionized water until they were taken out for compression testing.

4.2.3.3. Mechanical characterization of PA gels via AFM

Young’s moduli of the PA gels were determined via AFM (MFP-3D, Asylum Research Santa Barbara, CA) force-indentation curves (n = 15 – 45) acquired for each PA variant (n = 3 – 6) in a manner previously described (94, 127). First, cantilever spring constant and optical lever sensitivity of the AFM were calibrated against a glass slide (~50 GPa (188)) in ambient air contact mode. Then, deionized water was placed on top of the glass slide and the sensitivity of the optical path was re-calibrated in liquid contact mode. Next, a PA gel sample immersed in deionized water was mounted. A silicon nitride probe with a pyramidal tip and a nominal spring constant of 0.06 N/m (Bruker Corp., Camarillo, CA) was used to collect force-indentation profiles with an indentation depth and scan rate of 1-2 μm and 1-2 μm/s, respectively. Slower scan rates were chosen to minimize any viscous effects arising from probing in the liquid environment (193). We previously reported the Young’s moduli of the PA gels we fabricated using this approach (94).

Force curve data obtained from AFM indentations were analyzed with the Hertz cone model. Only the approach (forward indentation) curves of the force-indentation profiles were
used for analysis because retraction curves often exhibit high degrees of adhesive forces near the contact point (188). The AFM tip-sample interaction can be described by Hooke’s law, which relates the applied loading force \( F \) to the spring constant \( k \) and the deflection \( d \) of the cantilever. Since the cantilever deflection is equal to the position of the \( z \) piezo \( (z) \) minus the sample indentation \( (\delta) \), Hooke’s law can be written as follows (128, 194):

\[
F = k \times d = k(z - \delta)
\]  

(1)

Meanwhile, Hertz cone model relates the applied loading force to the sample indentation by the following equation (128, 194):

\[
F = \frac{2}{\pi} \delta^2 \tan \alpha \frac{E}{1-v^2}
\]  

(2)

\( E \) and \( v \) are the Young’s modulus and the Poisson ratio of the sample and \( \alpha \) is the half-opening angle of the cone (i.e., cantilever tip). Poisson ratio of the PA gels was assumed to be 0.4, consistent with the published range of 0.3-0.5 for PA substrates (127).

While it is possible to use the FIEL (force integration to equal limits) mapping to obtain relative elasticity information independent of the position of the contact point (188, 195), contact point determination is essential in order to acquire absolute elastic properties (189). An AFM force curve consists of the noncontact region and the contact region separated by the contact point \((z_0, d_0)\), which is the point when the cantilever tip first comes in contact with the sample (Fig. 4.1). Accurate determination of the contact point is therefore crucial to define the contact region and the resulting range of analysis. Incorporating the contact point into equations 1 and 2 yields the following (128, 194, 196).
Lin et al. summarized various strategies employed to determine the contact point (189). Visual inspection approach is the simplest available method but it generally provides a poor fit because the quality of the fit is not considered.

Alternative ways of determining the contact point include the constrained approach and the unconstrained approach (189, 197). The constrained approach refers to the strategy where the contact point is selected from the collected force dataset. This method utilizes the first and second derivatives of the cantilever deflection after smoothing the force curve, and can lead to large errors in very soft samples due to calculation of derivatives. The unconstrained approach, on the other hand, is where neither \( z_0 \) nor \( d_0 \) is fixed. Also known as the sequential or linear search schemes, this approach uses an iterative process to find the contact point with the best least-squares fit.

In this paper, we devised a variation of the unconstrained approach to determine the contact point in a simple manner. First, the zero deflection \( d_0 \) was estimated by fitting a linear line over the noncontact region. Next, a portion of the contact region or the ‘range of analysis’ bound by \( (z_1, d_1) \) and \( (z_2, d_2) \) was selected (Fig. 4.1). The range of analysis (128) defines the range of cantilever deflection values and therefore the range of applied forces where Hertz model is fitted to extract the Young’s modulus. Setting the range of analysis allowed the \( z \) piezo position at the point of contact \( (z_0) \) to be estimated using equation 3. Once the contact point \( (z_0, d_0) \), was determined, the AFM force data within the defined range of analysis was plotted as \( F \) vs. \( \delta^2 \). As in equation 2, the slope of this plot gives the Young’s modulus \( (E) \) of the sample. As a
default, the range of analysis of 10-30 nm was chosen for analysis because it provided a good fit over PA gels with a varying stiffness. To facilitate the processing of the large volume of the AFM force curve data, we wrote a Matlab code that calculates the Young’s modulus of the sample based on this approach.

4.2.3.4. Compression test

Conventional unconfined compression tests were used to determine the bulk elastic properties of the PA gels. PA gel discs were uniaxially compressed at a rate of 1.2 mm/min using a mechanical tester (Insight, MTS Systems) with a 250 N load cell. A stress vs. strain curve was plotted and the slope of the linear elastic region within the first 10% strain was used to calculate the Young’s modulus of the sample.

4.2.3.5. Statistical Analysis

All values are reported as average ± standard deviation.

4.2.4. Results

The Young’s moduli of the PA gels were determined independently via AFM and compression testing and the results were compared and summarized in Table 4.1. The resulting Young’s moduli of the samples were in good agreement for soft PA gels (%acrylamide/%bis-acrylamide: 5/0.10, 5/0.26). The values deviated from each other considerably for stiffer PA gels. The largest discrepancy was observed with the stiffest PA gel (38/2). Visually determined contact point provided a poor fit at this stiffness, with the Young’s modulus an order of magnitude smaller compared to others. Our unconstrained approach on the AFM force data
yielded a Young’s modulus that is triple the value obtained from compression testing. This difference may arise from the fact that AFM measures local elastic properties while compression testing measures bulk properties of the sample. Also, the geometry of the cantilever tip used in AFM indentations (pyramidal-shaped) may have overestimated the Young’s modulus. It was previously reported that sharp cantilever geometry tends to overestimate the sample stiffness compared to rounded cantilever geometry (i.e., pyramidal vs. spherical) (198). Wider tip geometry such as the spherical tip, however, can compromise the lateral resolution (199) and is more costly. For this reason, we chose a cantilever with a pyramid-shaped silicon nitride tip and used it consistently throughout our study.

Because our approach selected the contact point based on the chosen range of analysis, we examined the effect of the range of analysis on the resulting Young’s modulus of the sample. We varied the range of analysis within the first 50 nm of the cantilever deflection (5-10, 10-30, 30-50, 5-50 nm) for the select force curves for the soft (5/0.1) and stiff (38/2) PA gels and compared the resulting Young’s moduli (Table 4.2). The Young’s moduli estimated for the soft PA gel (5/0.1) varied from 2.74 kPa (30-50 nm) to 3.56 kPa (5-10 nm) or with ~30% disparity. The Young’s moduli for the stiff PA gel (38/2) varied from 2443.70 kPa (30-50 nm) to 5420.50 kPa (5-10 nm). These values suggested that a range of analysis should be close to the contact point but not too close (5-10 nm) or not too far (30-50 nm), as these tend to over- or underestimate the Young’s modulus. Therefore, we chose an intermediate range (10-30 nm) to analyze the AFM force data. Hertzian fits to the select force curves for the soft and stiff PA gels with this range of analysis are shown in Fig. 4.2.
**4.2.5. Discussion**

In recent years, the use of AFM as a nanoindentation tool to extract the Young’s modulus of soft sample has been extended to soft hydrogels and biological materials as the inhomogeneity or the change in the mechanics of the cellular microenvironment (23, 127) and the biological samples (194, 196, 200-203) themselves have been implicated in the regulation of cellular functions as well as pathological disease progression (188, 190). With the lateral resolution in the nm range, surpassing the limits of conventional fluorescence microscopy (~200 nm), AFM can be employed to study samples at the cellular and subcellular level (204).

AFM instrumentation and experimentation procedures as well as the application of the Hertz contact theory are subject to several sources of error and/or uncertainty in the calculation of the sample Young’s modulus. Hertz theory assumes linear elastic behavior with no adhesive interactions while the contact area between the tip and the sample is known (186-190). It also assumes that the tip shape, cantilever spring constant, and the viscoelastic property of the sample are known exactly, which is typically not the case. Another big source of error arises from the contact point estimation, which is necessary in determining the noncontact and contact regions of the AFM force curves. The contact point affects the selection of the range of analysis, and hence the Young’s modulus of the sample. Several mathematical models that account for surface forces and approaches to estimate the contact point have been proposed (189, 192), but they are often too rigorous to be applied for the routine analysis of the large-volume AFM force data.

To reduce errors and facilitate the processing of the AFM force data, we devised a simple calculation scheme based on the unconstrained approach to find the contact point. Comparing this approach to the visual inspection approach (AFM analysis) and mechanical compression testing showed that the resulting sample Young’s moduli were in good agreement for soft PA
gels. There was a large discrepancy for the stiff PA gel (38% acryl/2% bis-acryl) and the Young’s modulus calculated from our approach using the AFM force curve data was threefold the value obtained from compression testing. This is probably due to our choice of the AFM cantilever. The sharp tip geometry (pyramid) and small cantilever spring constant (nominal constant of 0.06 N/m) likely introduced large errors to the system and overestimated the local elastic properties of the stiff PA gels (38/2).

When we explored the effect of the range of analysis on the sample Young’s modulus, it became apparent that the Young’s modulus may be considerably over- or underestimated depending on the region selected. Therefore, it is crucial that a suitable range of analysis is chosen for analysis. For PA gels, 10-30 nm seems to provide good approximations of the sample Young’s modulus.

Due to the many potential sources of error and uncertainty in the Young’s modulus extraction from the AFM force data, reporting the sample Young’s modulus alone is not sufficient in comparing values obtained from different laboratories. An accurate characterization of the sample mechanical properties would therefore require an explicit report of the assumptions taken and the parameters used for data analysis. In particular, how the contact point was estimated and which range of analysis was chosen should be explicitly stated.

4.2.6. Conclusions

We implemented a simple approach to determine the contact point of AFM force curves to estimate the Young’s modulus of soft samples. Probing PA gels with a wide range of stiffness highlighted the importance of the contact point estimation method and the selection of the range of analysis. Our approach enables the quick processing of the large-volume AFM force data;
therefore, it will may useful for a range of bioengineering applications requiring mechanical analysis of soft samples.

4.3. Time-of-Flight Secondary Ion Mass Spectroscopy (TOF-SIMS)

4.3.1. Abstract

The ability to self-renew and differentiate into multiple types of blood and immune cells renders hematopoietic stem and progenitor cells (HSPCs) valuable for clinical treatment of hematopoietic pathologies and as models of stem cell differentiation for tissue engineering applications. To study directed HSC differentiation and identify the conditions that recreate the native bone marrow environment, combinatorial biomaterials that exhibit lateral variations in chemical and mechanical properties are employed. New experimental approaches are needed to facilitate correlating cell differentiation stage with location in the culture system. We demonstrate that multivariate analysis of time-of-flight secondary ion mass spectrometry (TOF-SIMS) data can be used to identify the differentiation state of individual hematopoietic cells (HCs) isolated from mouse bone marrow. Here, we identify primary HCs from three distinct stages of B cell lymphopoiesis at the single cell level: HSPCs, common lymphoid progenitors, and mature B cells. The differentiation state of individual HCs in a test set could be identified with a partial least squares discriminant analysis (PLS-DA) model that was constructed with calibration spectra from HCs of known differentiation status. The lowest error of identification was obtained when the intra-population spectral variation between the cells in the calibration and test sets was minimized. This approach complements the traditional methods that are used to identify HC differentiation stage. Further, the ability to gather mass spectrometry data from single HSCs cultured on graded biomaterial substrates may provide significant new insight into
how HSPCs respond to extrinsic cues as well as the molecular changes that occur during cell differentiation.

4.3.2. Introduction

The body’s full spectrum of blood and immune cells is generated from a small number of hematopoietic stem cells (HSCs) that are located within the bone marrow (36, 205-207). HSCs self-renew and differentiate into increasingly less-rare and more-mature HCs (206, 208-212). This renders HSCs of significant value for clinical treatment of hematopoietic pathologies and as models of stem cell differentiation (213).

Presently, much research focuses on developing culture systems that mimic the bone marrow’s chemotactic and micromechanical properties to enable control over HSC differentiation. Engineered biomaterials that exhibit spatial variations in mechanical properties and ligand presentation are especially attractive because they enable screening the effects of multiple microenvironments on HSC fate using a minimal number of cells (214-216). To utilize such combinatorial systems, rigorous methodologies to aid identification of HC differentiation stage at the single cell level with location specificity are highly attractive. Currently, differentiation stage is most commonly assessed via fluorescence microscopy using cocktails of differentiation stage specific antibodies (217); however ambiguity from single-cell fluorescence analysis of small cell populations and inter-user variability of immunolabeling approaches can be a significant drawback (45).

We hypothesized that information about the expression profiles of cell surface antigens as well as other differences in cell surface chemistry could be acquired with time-of-flight secondary ion mass spectrometry (TOF-SIMS), and exploited to identify the differentiation stage
of individual HCs within a culture. Mass spectral maps of the molecules at the surface of an individual cell can be collected with TOF-SIMS (218-220). Because the spectra collected from biomaterials using TOF-SIMS instruments equipped with liquid metal primary ion sources are dominated by low mass \((m/z<300)\) fragment ions that are common to multiple biomolecules, multivariate analysis is often employed to discriminate the spectra of biomolecules and cells (218, 221-232). Unknown samples have also been identified with supervised multivariate models that are constructed from the TOF-SIMS data of known samples (222, 229). For example, two different cell lines in a heterogeneous culture have been identified with location specificity by partial least-squares discriminant analysis (PLS-DA) of TOF-SIMS data (233). Though this work achieved the location-specific identification required for studies of HSC fate decision, the accuracy of identifying primary cells that exhibit higher intra-population heterogeneity than laboratory cells lines (234) with this approach has not been quantitatively assessed.

Here, we report our efforts to classify individual primary murine HCs isolated from the bone marrow according to their stage in the B lymphocyte differentiation pathway by multivariate analysis of TOF-SIMS data. We focus on identifying three populations of primary HCs that were isolated from murine bone marrow via conventional flow cytometry (Fig. 4.3): 1) hematopoietic stem and progenitor cells (HSPCs) that do not express lineage antigens (Lin-) but that do express Sca1 and cKit (Lin-Sca-1\(^+\)c-Kit\(^+\), LSK); 2) common lymphoid progenitors (CLPs, Lin-IL-7R\(\alpha\)Sca-1\(^{med}\)c-Kit\(^{med}\)); and 3) mature B cells (B220\(^+\)IgM\(^+\)) (131, 209). These populations represent distinct cell phenotypes during lymphopoiesis: uncommitted stem and progenitor cells (HSPCs), lineage specified progenitor cells (CLPs), and fully-differentiated cells (B cells). We further investigated use of TOF-SIMS data to identify differences between HSPC populations isolated from young and old mice. Though isolated using identical sorting criteria, significant
age-related differences in HSC functionality have been previously reported, making classification approaches that do not rely on surface antigen expression especially significant (235-237). The potential for existence of populational subfractions with improved HSPC stemness, therefore, motivated study of whether TOF-SIMS approaches could segregate HSPCs isolated via identical flow conditions from young and old mice.

Identification of the differentiation stage of individual primary HCs by multivariate analysis of TOF-SIMS data is complicated by the high degree of heterogeneity that exists within primary cell populations (56, 238, 239). Such intra-population heterogeneity can hinder the detection of the differentiation-related spectral features (221, 240). We selectively captured the spectral variation between, but not within, each cell population, by employing PLS-DA models constructed using spectra from HCs of known differentiation status (calibration data set) to accurately identify the differentiation state of test HC cells. The lowest error of identification was achieved when the intra-population spectral variance that may be caused by auto-specific and age-related differences in cell surface chemistry were minimized. This approach may enable identifying cell differentiation status and its relationship to location within a colony or engineered microenvironment.

4.3.3. Materials and Methods

4.3.3.1. HC isolation and preparation.

The hematopoietic subpopulations were isolated from the femoral and tibial bone marrow of female C57BL/6 mice (Jackson Labs). Distinct age ranges were used for ‘old’ (10 months old) and ‘young’ (2 – 4 months old) mice.
4.3.3.2. **TOF-SIMS.**

Mass spectral images were acquired in unbunched mode (total primary ion dose = 3 x 10^{13} ions/cm^2) using a PHI Trift-III TOF-SIMS (Physical Electronics Incorporated, Chanhassen, MN) instrument with a {^{197}Au}^+ liquid ion gun that was operated at 22 kV. The primary ion beam was raster scanned across the sample, and positive-ion spectra with a mass range of 0 to 800 amu were acquired at each pixel.

4.3.3.3. **Data analysis.**

Multivariate analysis was performed using the PLS Toolbox (v.6.2.1, Eigenvector Research, Manson, WA) run in MATLAB (v.7.12.0 R2011a, MathWorks Inc., Natick, MA). Unit mass binned spectra of individual cells were extracted from the TOF-SIMS images and imported into the PLS toolbox. Outlier spectra that exhibit unusual variation were identified (Fig. 4.4) and excluded from further analysis. Construction of the PLS-DA and PCA models is described in Fig. 4.5.

4.3.4. **Results**

4.3.4.1. **The Differentiation Status of Primary HCs Isolated from Mouse Bone Marrow Can Be Identified by PLS-DA of TOF-SIMS Data**

A PLS-DA model was constructed from a calibration data set consisting of the spectra acquired from 15 B cells, 13 CLPs, and 15 HSPCs isolated from five old mice. This PLS-DA model was then used to identify the differentiation stage of 15 B cells, 12 CLPs, and 15 HSPCs (test data set) also harvested from the same mice. To increase the probability that the identification was based on cell surface biomolecules and not contaminants, only the peaks that
were related to amino acids, phosphocholine, and fatty acids were analyzed (cell-related peak set, Table 4.3 (240, 241). Fig. 4.6(A)-(C) shows the identifications made with the PLS-DA model, where the cells that exceeded the classification threshold (red dashed lines in Fig. 4.6(A)-(C)) were identified as the indicated population. Table 4.3 lists the sensitivity (the fraction of cells in the specified population correctly identified as that population), specificity (the fraction of cells from other populations that were correctly identified as not in the specified population) and error (average of the false positive and false negative rates) for identifying each HC population. The differentiation stages of the calibration cells were re-identified with high sensitivity and specificity, and the errors for identifying the B cells, CLPs, and HSPCs in the calibration set were only 0%, 2%, and 10%, respectively. Likewise, the differentiation stages of the cells in the test set were also identified with high sensitivity, high selectivity, and low error (3%, 8%, and 11% identification error for the test B cells, CLPs, and HSPCs, respectively). Thus, the variance in the peaks related to amino acids, phosphocholine, and fatty acids in the spectra was characteristic of HC populations, and could be exploited to identify the differentiation stage of individual HCs.

The contributions of each mass peak to the spectral variance that identifies the B cells, CLPs, and HSPCs are shown in the variable importance in projection (VIP) plots (Fig. 4.6(D)-(F)). Peaks with VIP scores greater than unity exhibit variance that is important towards identifying the indicated population (242, 243). For at least two of the three cell types, peaks m/z 53, 55, 86, 130, 148, 166, 184, 190, 205, 206, 210, and 279 have high VIP scores. Although some of these peaks are related to multiple biomolecular building blocks, phosphocholine, fatty acids, glutamine, glutamic acid, leucine, and tryptophan are likely candidates for the parent components because they are related to two or more of the peaks with high VIP scores. The
amino acid-related peaks with high VIP scores might reflect changes in the differentiation-specific proteins expressed on the cell surface, or the presence of the different antibodies used to isolate each HC population by flow cytometry. To investigate whether the antibodies used for cell isolation significantly contributed to cell identification, a PLS-DA model was constructed using spectra from the antibody cocktails that were used to isolate each HC population. Few of the peaks with the highest importance towards identifying the B cells, CLPs, and HSPCs (Fig. 4.6(D)-(F)) also had high importance towards identifying the antibodies used to isolate the B cells, CLPs, and HSPCs (Fig. 4.5), respectively. This suggests that the mass fragments produced by the population-specific antibodies did not contribute significantly to the spectral variation that identified each HC population. Though analysis of only the peaks related to amino acids, phosphocholine, and fatty acids increases the probability that the identifications are based on biomolecules and not sample-specific contaminants, it precludes detecting differentiation-related variations in other cell surface components, such as glycans (244, 245). Higher sensitivity and specificity of identifying the calibration and test cells was achieved when PLS-DA was performed using all of the peaks between 50 and 300 m/z that were not related to known contaminants (Table 4.3 and Fig. 4.5). However, the resulting model may not be applicable towards identifying the differentiation stage of other cell samples if the peaks with high VIP scores were related to sample-specific contaminants, and not cell biomolecules.

4.3.4.2. Extent of Intra-Population Variation between HCs from Mice that Differ in Age

This approach to identifying HC differentiation status would have greater applicability if the PLS-DA model could be constructed using the spectra of cells that were harvested from different mice as those in the test set. However, primary cells from different mice exhibit auto-
specific and age-related differences in cell surface chemistry (234, 235). Though subtle, these differences may increase the intra-population spectral variance between the calibration and test cells to a level that is detrimental to identifying cell differentiation stage. To investigate whether such changes in cell surface chemistry induce detectable spectral variance within each HC population, PCA was performed on the spectra of cells that were harvested from two sets of C57BL/6 mice that differed in age: 10-month-old (old) and 2- to 4-month-old (young) mice. These two age groups were selected because HSCs from mice of these ages exhibit identical surface antigen expression but significant functional and epigenetic differences (235-237). Note that the intra-population spectral variation detected between these old and young cells is likely larger than that exhibited by the cells that are used to study HSC fate decisions (typically < 6 months of age) (56, 93, 103, 246, 247).

The B cells from the old and young mice were not separated on the first principal component (PC) of the PCA model (Fig. 4.5). This indicates the linear combination of mass peaks whose intensities varied the most (26%) within this B cell population was not caused by auto-specific or age-related changes in cell chemistry. However, the B cell spectra from the young and old mice were separated on PC2 and PC3; the young B cells had positive scores on PC2 and PC3, whereas the old B cells had negative scores on PC2 and/or PC3 (Fig. 4.7(A)). The majority of the peaks with high negative loadings on both PC2 and PC3, and therefore, higher normalized intensities in the spectra of the B cells from the old mice, were mainly related to lipids (m/z 86, 166, 168, 182, 184, and 224). In contrast, many of the peaks with high positive loadings on PC2 and PC3, and higher normalized intensities in the spectra of B cells from the young mice, were related to amino acids (m/z 51, 130, 155, 178, 179, 205, 263, and 279). This
suggests that the B cells from the young mice had a higher ratio of proteins to lipids on their surfaces than the B cells from the old mice.

For the PCA models constructed for the CLPs and HSPCs, the first PCs captured approximately half of the spectral variance in the data set and separated the spectra of cells from the old and young mice (Fig. 4.7(B), (C)). Thus, the major source of spectral variation in the HSPC and CLP populations could be attributed to age-related and auto-specific differences in cell surface chemistry. In both models, the CLPs and HSPCs from the young mice had positive scores on PC1 and PC2, whereas the CLPs and HSPCs from the old mice had negative scores on PC1 and/or PC2. The peaks with high negative loadings on PC1 and PC2 were mainly related to lipids, whereas peaks that were mainly related to amino acids had high positive loadings on these two PCs (Fig. 4.7(B), (C)). Thus, like the B cells, the surfaces of the CLPs and HSPCs from the old mice have lower protein to lipid ratios than the surfaces of the CLPs and HSPCs from the young mice.

Though a more extensive study that employs a larger number of mice would be required to confirm these results, this preliminary data suggests that all three HC populations exhibit an age-related decrease in the protein to lipid ratio on the cell surface. This finding is consistent with previous work that demonstrated glycerolipid metabolism increases in the tissues of aged mice (248). However, complementary metabolic profiling analyses would be required to exclude the possibility that age-related increases in protein misfolding lead to an increase in the degradation or intracellular accumulation of protein (249), and a decrease in the protein to lipid ratio on the aged HCs.
4.3.4.3. Effects of Intra-Population Variation between the Calibration and Test Spectra on the Identification of HC Differentiation Stage

We next assessed whether the intra-population spectral variance detected with PCA was significant enough to compromise the identification of HC differentiation stage. The spectra of 30 B cells, 25 CLPs, and 29 HSPCs that were harvested from the old mice were used to construct a PLS-DA model that was used to identify the differentiation stage of the 20 B cells, 20 CLPs, and 14 HSPCs in the test data set from the young mice. Self-identification of the old B cells, CLPs, and HSPCs in the calibration set was achieved with ≤4% error (Fig. 4.8(A)-(C), Table 4.5), demonstrating the variance between the calibration cells was well-captured by the model. The peaks with high VIP scores (Fig. 4.8(D)-(F)) were similar to those in Fig. 4.6(D)-(F). Identification of the differentiation stage of the test HCs from the young mice using this model was less accurate, as the errors for identifying the test B cells, CLPs, and HSPCs from the young mice were 19%, 26% and 11%, respectively (Table 4.5). Thus, auto-specific and age-related differences in the calibration and test spectra appeared to compromise the identification of HC differentiation stage.

Finally, we investigated whether use of the complete peak set improved the precision of identifying HC differentiation stage. The errors in identifying the calibration cells were ≤6% (Table 4.5), respectively, which is similar to that achieved with the cell-related PLS-DA model. Inclusion of the mass peaks that were related to unknown biomolecules in the analysis was detrimental to identifying the differentiation status of the test cells from the young mice; the prediction error rose to 34%, 26%, and 49% for the test B cells, CLPs, and HSPCs, respectively (Table 4.5, Fig. 4.9). Thus, the peaks not related to amino acids, phosphocholine, or fatty acids varied significantly between the spectra in the calibration and test sets. Additional work towards
identifying the parent molecules that produced the unknown peaks with high VIP scores is required to determine whether the spectral variation between the calibration and test cells was due to contaminant molecules or changes in cell surface chemistry.

4.3.5. Discussion and Conclusions

Biomaterial substrates that exhibit orthogonal gradients in composition and stiffness have the potential to permit elucidation of the combination of cues that induce specific HSC fates. The rarity of these cells in the body makes quantitative single cell analysis methods particularly valuable. To realize this potential, robust methods must be developed to identify the differentiation stages of individual HSCs at distinct regions on a biomaterial. For this purpose, we have shown that TOF-SIMS data encodes for the surface chemistries exhibited by distinct HC populations, and PLS-DA can translate this chemical data into HC differentiation stage. Because PLS-DA uses numerical algorithms to quantify the probability that a HC is at the specified differentiation stage, this approach is more objective and less prone to inter-user variability than traditional immunolabeling methods. Additionally, cell surface chemistries that may be distinctive of individual organisms or aging are also encrypted in the TOF-SIMS data. Differences between the auto-specific and age-related surface chemistries exhibited by the cells in the calibration and test sets induce spectral variance that compromises the identification of HC differentiation stage. Consequently, the cells employed to construct the PLS-DA model should be from the same age group as the unknown cells in the test set to optimize precision. Having demonstrated the feasibility of identifying the differentiation stages of individual primary HCs with location specificity by multivariate analysis of TOF-SIMS data, we expect ongoing work may enable the identification of additional HC subpopulations. To improve our capacity to
detect subtle changes in HC phenotype, we are currently incorporating additional distinct HC subpopulations to construct a next generation of PLS-DA models. Most significantly, the HSPC population used in the current analyses contains a mix of stem and progenitor cells with differential long-term stem cell potential (Fig. 4.3). Future work that examines significantly more rare hematopoietic subpopulations (i.e. LSK CD150^+CD244^-CD48^-) (45) that are specifically enriched for the most primitive HSCs subpopulations and show significant age-related changes in stemness (247) would likely enhance our ability to identify early HSC fate decisions. Overall, we expect that use of this approach to identify the differentiation stages of individual HCs within combinatorial biomaterials will greatly facilitate correlating HSC fate decisions to environmental cues, critical to the design of *ex vivo* culture systems in order to control HSC bioactivity.

Our data indicate that the ratio of proteins to lipids on the surfaces of B cells, CLPs, and HSPCs decreases as the age of the donor mouse increases. Though we limited discussion of this observation to comparison with current knowledge in the field, this finding demonstrates that information on the cell surface chemistries that differ between HC populations may be acquired with this approach. Presently, the amount of compositional information that can be extracted from the data presented herein is restricted by two factors; our ability to ascertain the origins of the peaks with high importance towards identifying each HC population, and the quality of the mass spectra. Published databases of TOF-SIMS peaks that are related to lipids (240), amino acids (218, 231), and nucleobases (250) facilitate interpreting TOF-SIMS data. Identification of the mass peaks associated with glycans, cholesterol, and other cell surface molecules would also aid this effort. The low mass range that could be detected with our instrumentation and use of unit mass binning, which reduced the mass resolution such that multiple molecules likely
contributed to each spectral peak, ultimately limit the compositional information that might be extricated from our data. Use of a TOF-SIMS instrument with a cluster ion source that enhances the yields of high mass ions (251) and a mass spectrometer with higher mass resolving power and sensitivity would greatly enhance interpreting the population-specific spectral variance and identifying HC differentiation stage. Alternatively, a MALDI-TOF with sufficient spatial resolution to analyze individual cells may enable the more sensitive acquisition of mass spectra with a wider mass range and higher mass resolution from individual HCs. We expect that with the aforementioned improvements in technology and databases, multivariate analysis of TOF-SIMS data may also enhance efforts to elucidate the biomolecular changes that occur during differentiation or accompany age-related deficits in HC function.

4.4. Raman Spectroscopy

4.4.1. Abstract

Raman spectroscopy is able to resolve low-frequency vibrational modes in sample specimen via inelastic scattering of laser light. Increasingly, Raman approaches are believed to have potential as single cell analysis tools that may allow rapid, non-invasive, label-free screening of biological samples in ex vivo conditions (253). For example, live cells in their native cell culture media can be analyzed directly, making it an attractive option for in situ analysis (254). The use of laser light is a drawback, but previous reports suggest that such exposure particularly at longer wavelengths (e.g., 785 nm) has negligible effect on cells due to relatively short exposure time (255). Raman spectra obtained from cells could provide compositional information of single, individual cells regarding endogenous biomolecular content such as proteins, nucleic acids, and carbohydrates (256). This information can be resolved to the point
that it can be specific to the cell(s) analyzed, therefore providing characteristic information that could be used to identify cell types and their proliferation states (253). Here, we gathered Raman spectra of fixed cells within hematopoietic lineage to analyze subsets of stem cells (LT-HSC vs. ST-HSC) and mature cells of lymphoid (B cells) and myeloid (Gr-1+ granulocytes) lineages and used the information to successfully identify them. Ongoing work is analyzing live cells of hematopoietic lineage in an in vitro culture at varying stages of differentiation to assess the feasibility of Raman spectroscopy as a new functional metric for HSCs and lineage-restricted progenitors.

4.4.2. Materials and Methods

4.4.2.1. Preparation of the substrates

Polyacrylamide (PA) gels (12-mm diameter) were fabricated on top of 12.7-mm diameter glass-protected gold mirrors (Thor Labs, Inc., Newton, NJ) and coated with fibronectin following previously reported fabrication techniques (94, 122, 124). In brief, 4M NaOH was placed on top of glass-protected gold mirrors at 60 °C for 45 minutes. Gold mirrors were then washed, aminosilanized, and incubated with acrylamide/bis-acrylamide mixture for polymerization. Once polymerization was complete, PA gels were functionalized with fibronectin (100 µg/mL) by covering the surface with 50 mM Sulfo-SANPAH and exposing it to UV (365-nm; UVP, Upland, CA).

4.4.2.2. Isolation of hematopoietic cells

C57BL6 mice (The Jackson Laboratory, Bar Harbor, ME) between 4 and 8 weeks of age were euthanized with carbon dioxide in compliance with the University of Illinois Institutional
Animal Care and Use Committee (IACUC) guidelines. Their femurs and tibias were gathered, crushed, and filtered through a 40-μm cell strainer (BD Falcon, Franklin Lakes, NJ) in PBS supplemented with 2% FBS (buffer) to collect whole bone marrow (WBM) cells. Red blood cells were removed by ACK lysis buffer. To prevent nonspecific binding, WBM cells were incubated with Fc receptor blocking antibody (CD16/CD32). They were then incubated with a cocktail of FITC-conjugated lineage (Lin) antibodies (CD5, CD45R (B220), CD11b, Anti-Gr-1 (Ly-6G/C), 7-4, Ter-119), PE-conjugated Sca-1, APC-Cy7-conjugated c-Kit, and APC-conjugated CD34 to isolate CD34^+LSK cells (LT-HSC) and CD34^+LSK cells (ST-HSC). Similarly, cells were incubated with FITC-conjugated B220 and eFluor 450-conjugated IgM to isolate B cells (B220^+IgM^+) and with FITC-conjugated Gr-1 to isolate Gr-1^+ granulocytes. Propidium iodide was used to exclude dead cells. Labeled cells were sorted with FACS (BD FACSAria™ cell sorter) to isolate appropriate cell populations. All antibodies were purchased from eBiosciences, San Diego, CA. All other reagents were purchased from Life Technologies, Grand Island, NY unless otherwise noted.

4.4.2.3. Raman Spectra Analysis

Isolated cells were collected onto PA gels on gold mirrors and analyzed with a Raman confocal microscope immediate after or following in vitro culture as previously described (253). Prior to analysis, cells were fixed in 2% paraformaldehyde and 0.05% glutaraldehyde diluted in 0.2 M Hendry's phosphate buffer (HPB) for 30 min. Raman spectra were obtained with a Raman confocal microscope (Horiba LabRAM HR 3D confocal Raman imaging system) at room temperature using a Leica 50x, NA 0.50, objective with a working distance of 10.6 mm for fixed-cell experiments. A 350 mW and 785 nm laser was used to focus on a spot that was 10 to
15 μm in diameter on each cell and adjacent non-cellular material and neighboring cells were excluded from the focal area. The resulting cell spectra were adjusted to subtract background noise and analyzed to construct a partial least-squares discriminant analysis (PLS-DA) model and apply principal component analysis (PCA).

4.4.3. Preliminary Results

Raman spectra of LT-HSC, ST-HSC, and committed cells of lymphoid (B cells) and myeloid (Gr-1+ granulocytes) lineages were obtained (Fig. 4.10). Based on these, a PLS-DA model was constructed (Fig. 4.11) and principal component analysis (Fig 4.12) was performed to reveal significance compositional differences in analyzed cells. This approach was able to successfully identify LT-HSC, ST-HSC, B cells, and granulocytes. Cell cycle-related information was also extracted from the Raman spectra obtained, which showed more active replication activity (more cells in S phase) in HSCs compared to mature cells (Fig. 4.13). Raman spectra could further detect induced changed in cell cycle (Fig. 4.14). Here, adding SCF increased proliferation as indicated by more cells being in the S phase.

4.4.4. Ongoing Work

Preliminary results show that Raman spectroscopy has potential to be used as a new functional metric for cells of hematopoietic lineage, from HSCs to lineage-restricted progenitors to mature cells. To further establish its feasibility, ongoing work is investigating Raman analysis of live cells immediately after isolation or after culturing them short-term in vitro. For the latter case, cells will be cultured on substrates with varying stiffness identified in Chapter 3 of this
thesis to impact HSC fate in order to confirm that Raman spectroscopy is sensitive for the detection of any subtle changes induced by microenvironmental biophysical cues.
4.5. Figures

**Figure 4.1. Schematic of an AFM force curve.**
This graph shows what a typical AFM force-indentation curve looks like. Only the extraction curve is used for analysis. Important features include contact and non-contact region, range of analysis, and the contact point. The point when the tip first contacts the sample surface is the contact point. The range of analysis defines the set of deflection and therefore applied force values within the contact region where the Hertz model is applied.
Figure 4.2. AFM force-indentation curves.
Force-indentation curves for soft (5% acryl/0.1% bis) and stiff (38% acryl/2% bis) PA gels from AFM measurements (blue) and Hertz model-based theoretical fits over the range of analysis 10-30 nm (black) are shown.
Figure 4.3. Cellular constituents of HSC-mediated hematopoiesis.
The blue, green, and red envelopes indicate the cell populations used in this study. The HSPC population (Lin−Scal−cKit+) used in our study (blue envelope) contains long-term HSCs (LT-HSCs) capable of sustained hematopoietic reconstitution, short-term HSCs (ST-HSCs) capable of limited hematopoietic constitution, and multipotent progenitors (MPPs) which retain lymphoid/myeloid lineage plasticity. B cell lymphopoiesis is marked by MPP progression to a common lymphoid progenitor (CLP, green envelope) cell capable of generating all T lymphocytes, B lymphocytes, dendritic cells (DC), and natural killer (NK) cells. B lymphopoiesis further progresses through a sequence of defined precursor populations: pre-pro-B cell, pro-B cell, pre-B cell, immature-B cell (Im-B), and finally mature B cell (red envelope) (209). Additional cell constituents depicted: CMP, common myeloid progenitor; MEP, megakaryotic/erythroid progenitor; GMP, granulocyte/monocyte progenitor. Image modified from Passegue et al (252).
Figure 4.4. Identification of outlier spectra from cells harvested from the old (A) and young (B) mice.
Using the spectra acquired for each group of cells, a PC model with two principal components was constructed using the mass peaks in the 1–300 m/z range. Outlier samples exhibited spectral variation that was not captured by the PCA model. The outlier spectra (indicated with black arrows) have Q residual values that are greater than the 95% confidence interval (horizontal dashed line). These samples were removed from the data set and excluded from further analysis.
Figure 4.5. Construction of the PLS-DA model.

(A) Plots show the contributions of the peaks in the cell-related peak set to the overall Q residual and Hotelling’s $T^2$ statistic in the preliminary PLS-DA models. Peaks with high Q residual contributions and low Hotelling’s $T^2$ contributions vary in a manner that is not related to HC differentiation status. (A) The preliminary PLS-DA model that was used to test the differentiation status of cells that were harvested from the same aged mice as the calibration set was refined by excluding the mass peaks with Q contributions above 30% (red). (B) The preliminary PLS-DA model that was used to test the differentiation status of cells that were harvested from young mice was refined by excluding the mass peaks with Q contributions above 60% (red).

(B) The VIP score plots show the importance of each mass peak towards the identification of the (A) B cell-specific antibodies, (B) CLP-specific antibodies, and (C) HSPC-specific antibodies that were used to isolate each cell population using flow cytometry.

(C) The (A) B cell, (B) CLP, and (C) HSPC identification plots for the PLS-DA model constructed using the complete peak set show the cells that exceed the threshold (red dashed line) for identification as the indicated cell type. The VIP plots for the (D) B cells, (E) CLPs, and (F) HSPCs show that many peaks from unknown sources were important towards identifying the indicated population (purple peaks).

(D) (A) PC score plot shows that PC1 captured the largest percentage of spectral variation in the B cell spectra, but did not separate the B cell spectra according to the age of the mice that the cells were harvested from. The elliptical region that is outlined with the dashed blue line represents the 95% confidence limit of the PC model. (B) The loadings plot shows the contribution of each peak to the variation captured by each PC.
Figure 4.6. Identification plots and VIP score plots for the PLS-DA models.

PLS-DA models were constructed using the cell-related peaks in the calibration spectra of HCs that were harvested from the same mice as the cells in the test set. The cells that exceeded the classification threshold (red dashed line) in the prediction plots were identified as (A) B cells, (B) CLPs, and (C) HSPCs. The VIP score plots for this model show the importance of each mass peak towards the identification of the (D) B cells, (E) CLPs, and (F) HSPCs. Peaks with VIP scores greater than unity are important for identifying the indicated population.
Figure 4.7. PC score and loadings plots for HCs from young and old mice.
PC score and loadings plots were constructed using the spectra from the B cells (A), CLPs (B), and HSPCs (C) that were harvested from the old and young mice. The region within the dashed blue line on each score plot represents the border for the 95% confidence limit of the entire PC model. The loading plots for each cell type show the extent that each mass peak contributed to the variance captured by the indicated PC.
Figure 4.8. HCs from young mice were identified using PLS-DA model.
The differentiation stages of HCs harvested from young mice were identified using a PLS-DA model that was created using the cell-related peaks in a calibration set of spectra from HCs harvested from old mice. The cells that exceeded the threshold (red dashed line) were identified as (A) B cells, (B) CLPs, and (C) HSPCs. The VIP score plots show the importance of each mass peak towards the identification of the (D) B cells, (E) CLPs, and (F) HSPCs.
Figure 4.9. Identification plots for PLS-DA model constructed using the complete peak set. The (G) B cell, (H) CLP, and (I) HSPC identification plots for the PLS-DA model that was constructed using the complete peak set show that the inclusion of the peaks from unknown sources was detrimental to identifying each cell population. The VIP plots for the (J) B cells, (K) CLPs, and (L) HSPCs shows that many of the peaks that were important towards identifying the cells in each population were not included in the cell-related peak set (purple peaks). The parent molecules that produced these fragments are not known.
Figure 4.10. Raman spectra of primary hematopoietic cells.
Raman spectra of primary LT-HSC (CD34^−LSK), ST-HSC (CD34^+LSK), B cell (IgM^+B220^+), and granulocyte (Gr-1^+) are shown here.
**Figure 4.11. PLS-DA model.**

Based on Raman spectra, PLS-DA model was constructed to identify primary LT-HSCs (CD34^-LSK), ST-HSCs (CD34^-LSK), B cells (IgM^+B220^+), and granulocytes (Gr^-1^+). Latent variable 1 (LV1) accounts for DNA and RNA-related peaks.
Figure 4.12. PCA of the obtained Raman spectra.
In addition to PLS-DA model, PCA was applied to cluster Raman spectra of hematopoietic cell populations.
Figure 4.13. Cell cycle information from Raman Spectra.
Raman spectra could be used to resolve cell cycle information of individual cells. Cell cycle data suggests that hematopoietic stem and progenitor cells (CD34+ LSK cells) have higher fractions of cells replicating (in S phase) than mature cells (B cells, granulocytes).
Figure 4.14. Cytokine-induced changes in cell cycling status of CD34+LSK cells.
Hematopoietic stem and progenitor cells (CD34+LSK cells) cultured with cytokines (100 ng/mL of SCF, TPO, Flt3L) showed higher fractions of cells replicating (in S phase) than those cultured without.
4.6. Tables

Table 4.1. Young's modulus of the PA gels estimated via AFM and compression testing.

<table>
<thead>
<tr>
<th>% Acrylamide/ % Bis-Acrylamide</th>
<th>AFM</th>
<th>Compression Testing</th>
<th>Compression Testing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$E$ (kPa) Visual Inspection Approach (94)</td>
<td>$E$ (kPa) Unconstrained Approach</td>
<td>$E$ (kPa)</td>
</tr>
<tr>
<td>5/0.10</td>
<td>3.48 ± 0.59</td>
<td>3.13 ± 0.40</td>
<td>4.73 ± 1.00</td>
</tr>
<tr>
<td>5/0.26</td>
<td>8.95 ± 1.40*</td>
<td>8.42 ± 1.11</td>
<td>--</td>
</tr>
<tr>
<td>10/0.3</td>
<td>--</td>
<td>51.2 ± 16.1</td>
<td>32.4 ± 10.9</td>
</tr>
<tr>
<td>15/0.6</td>
<td>--</td>
<td>227 ± 23.4</td>
<td>125 ± 56.5</td>
</tr>
<tr>
<td>38/2</td>
<td>196 ± 54.1</td>
<td>3756 ± 803</td>
<td>1236 ± 299</td>
</tr>
</tbody>
</table>

*: Additional force curves were analyzed for this paper
**Table 4.2.** Young’s modulus of the PA gels estimated over varying regions of analysis.

<table>
<thead>
<tr>
<th>Range of analysis</th>
<th>5% acryl/ 0.1%bis $E$ (kPa)</th>
<th>38% acryl/ 2%bis $E$ (kPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 - 10 nm</td>
<td>3.56</td>
<td>5420.50</td>
</tr>
<tr>
<td>10 - 30 nm</td>
<td>3.17</td>
<td>3518.70</td>
</tr>
<tr>
<td>30 - 50 nm</td>
<td>2.74</td>
<td>2443.70</td>
</tr>
<tr>
<td>5 - 50 nm</td>
<td>3.12</td>
<td>3338.60</td>
</tr>
</tbody>
</table>
Table 4.3. Known mass peaks for TOF-SIMS analysis.
List of the mass peaks in the cell-specific peak set and the biomolecules that are related to these peaks (240) (218). Peaks that are produced by common surface contaminants were omitted (241).

<table>
<thead>
<tr>
<th>Mass (m/z)</th>
<th>Related Biomolecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>51</td>
<td>phenylalanine</td>
</tr>
<tr>
<td>53</td>
<td>lipid (C₆H₆N⁺)</td>
</tr>
<tr>
<td>54</td>
<td>lipid fragment (¹³C²C₃H₄⁺)</td>
</tr>
<tr>
<td>55</td>
<td>lipid fragment (C₆H₇⁺), arginine, lysine, cysteine, valine, leucine, histidine</td>
</tr>
<tr>
<td>56</td>
<td>lipid fragment (C₆H₈N⁺), lysine, methionine, glutamine, asparagine, threonine, isoleucine</td>
</tr>
<tr>
<td>57</td>
<td>lipid fragment (C₆H₈⁺), valine, serine, alanine, arginine, cysteine, aspartic acid, leucine, threonine, glycine</td>
</tr>
<tr>
<td>58</td>
<td>lipid fragment (C₆H₈N⁺), isoleucine</td>
</tr>
<tr>
<td>59</td>
<td>lipid fragment (¹³C²C₃H₈N⁺), arginine, valine</td>
</tr>
<tr>
<td>60</td>
<td>lipid fragment (C₆H₁₀N⁺), serine</td>
</tr>
<tr>
<td>61</td>
<td>methionine</td>
</tr>
<tr>
<td>62</td>
<td>lipid fragment (C₆H₆⁺)</td>
</tr>
<tr>
<td>63</td>
<td>lipid fragment (C₆H₈N⁺), proline</td>
</tr>
<tr>
<td>64</td>
<td>lipid fragment (C₆H₇⁺), isoleucine, histidine, lysine</td>
</tr>
<tr>
<td>65</td>
<td>lipid fragment (C₆H₈N⁺), proline, leucine, glutamic acid, asparagine, arginine</td>
</tr>
<tr>
<td>66</td>
<td>lipid fragment (C₆H₆⁺)</td>
</tr>
<tr>
<td>67</td>
<td>lipid fragment (C₆H₈⁺), alanine</td>
</tr>
<tr>
<td>68</td>
<td>lipid fragment (C₆H₈N⁺), histidine</td>
</tr>
<tr>
<td>69</td>
<td>lipid fragment (C₆H₇⁺)</td>
</tr>
<tr>
<td>70</td>
<td>lipid fragment (C₆H₈N⁺), lysine, glutamine, glutamic acid</td>
</tr>
<tr>
<td>71</td>
<td>lipid fragment (C₆H₇⁺)</td>
</tr>
<tr>
<td>72</td>
<td>lipid fragment (C₆H₈N⁺), valine</td>
</tr>
<tr>
<td>73</td>
<td>lipid fragment (C₆H₇⁺)</td>
</tr>
<tr>
<td>74</td>
<td>cysteine, glycine</td>
</tr>
<tr>
<td>75</td>
<td>phenylalanine</td>
</tr>
<tr>
<td>76</td>
<td>lipid fragment (C₆H₆⁺), alanine</td>
</tr>
<tr>
<td>77</td>
<td>lipid fragment (C₆H₆⁺), serine, phenylalanine, methionine</td>
</tr>
<tr>
<td>78</td>
<td>lipid fragment (C₆H₇⁺)</td>
</tr>
<tr>
<td>79</td>
<td>lipid fragment (C₆H₇⁺)</td>
</tr>
<tr>
<td>80</td>
<td>lipid fragment (C₆H₈N⁺), histidine</td>
</tr>
<tr>
<td>81</td>
<td>lipid fragment (C₆H₇⁺)</td>
</tr>
<tr>
<td>82</td>
<td>lipid fragment (C₆H₈N⁺), glutamic acid</td>
</tr>
<tr>
<td>83</td>
<td>lipid fragment (C₆H₇⁺)</td>
</tr>
<tr>
<td>84</td>
<td>lipid fragment (C₆H₈N⁺), lysine, glutamine, glutamic acid</td>
</tr>
<tr>
<td>85</td>
<td>lipid fragment (C₆H₇⁺)</td>
</tr>
<tr>
<td>86</td>
<td>lipid fragment (C₆H₈N⁺), isoleucine, leucine</td>
</tr>
<tr>
<td>87</td>
<td>asparagine</td>
</tr>
<tr>
<td>88</td>
<td>lipid fragment (C₆H₈N⁺), aspartic acid</td>
</tr>
<tr>
<td>89</td>
<td>alanine</td>
</tr>
<tr>
<td>90</td>
<td>lipid fragment (C₆H₆⁺), serine, phenylalanine, methionine</td>
</tr>
<tr>
<td>91</td>
<td>lipid fragment (C₆H₆⁺)</td>
</tr>
<tr>
<td>92</td>
<td>lipid fragment (C₆H₇⁺)</td>
</tr>
<tr>
<td>93</td>
<td>lipid fragment (C₆H₇⁺)</td>
</tr>
<tr>
<td>94</td>
<td>lipid fragment (C₆H₇⁺)</td>
</tr>
<tr>
<td>95</td>
<td>lipid fragment (C₆H₇⁺), histidine</td>
</tr>
<tr>
<td>96</td>
<td>lipid fragment (C₆H₇⁺)</td>
</tr>
<tr>
<td>97</td>
<td>lipid fragment (C₆H₈⁺)</td>
</tr>
<tr>
<td>98</td>
<td>lipid fragment (C₆H₈⁺), glycine</td>
</tr>
<tr>
<td>99</td>
<td>lipid fragment (C₆H₈⁺)</td>
</tr>
<tr>
<td>100</td>
<td>lipid fragment (C₆H₁₀N⁺)</td>
</tr>
<tr>
<td>101</td>
<td>glutamine</td>
</tr>
<tr>
<td>102</td>
<td>lipid fragment (C₆H₈NO⁺), glutamic acid</td>
</tr>
<tr>
<td>103</td>
<td>phenylalanine</td>
</tr>
<tr>
<td>104</td>
<td>lipid fragment (C₆H₁₀NO⁺), lysine</td>
</tr>
<tr>
<td>105</td>
<td>serine</td>
</tr>
<tr>
<td>106</td>
<td>tyrosine</td>
</tr>
<tr>
<td>107</td>
<td>histidine</td>
</tr>
<tr>
<td>108</td>
<td>lipid fragment (C₆H₈⁺)</td>
</tr>
<tr>
<td>109</td>
<td>alanine</td>
</tr>
<tr>
<td>116</td>
<td>serine, tyrosine, threonine, proline</td>
</tr>
<tr>
<td>117</td>
<td>tryptophan, proline</td>
</tr>
<tr>
<td>118</td>
<td>valine</td>
</tr>
<tr>
<td>120</td>
<td>phenylalanine, methionine, threonine, glycine</td>
</tr>
<tr>
<td>122</td>
<td>cysteine</td>
</tr>
<tr>
<td>123</td>
<td>tyrosine</td>
</tr>
<tr>
<td>128</td>
<td>serine</td>
</tr>
<tr>
<td>130</td>
<td>glutamine, tryptophan, glutamic acid</td>
</tr>
<tr>
<td>132</td>
<td>isoleucine, leucine</td>
</tr>
<tr>
<td>133</td>
<td>asparagine</td>
</tr>
<tr>
<td>136</td>
<td>alanine, aspartic acid</td>
</tr>
<tr>
<td>138</td>
<td>proline</td>
</tr>
<tr>
<td>143</td>
<td>tryptophan</td>
</tr>
<tr>
<td>146</td>
<td>lipid fragment (C(_5)H(_9)NPO(_2^+))</td>
</tr>
<tr>
<td>148</td>
<td>lipid fragment (C(_5)H(_9)NPO(_2^+)), glutamic acid</td>
</tr>
<tr>
<td>150</td>
<td>lipid fragment (C(_5)H(_9)NPO(_2^+)), methionine</td>
</tr>
<tr>
<td>155</td>
<td>asparagine, aspartic acid</td>
</tr>
<tr>
<td>156</td>
<td>histidine</td>
</tr>
<tr>
<td>159</td>
<td>tryptophan</td>
</tr>
<tr>
<td>165</td>
<td>tyrosine</td>
</tr>
<tr>
<td>166</td>
<td>lipid fragment (C(_5)H(_9)NPO(_2^+)), methionine, phenylalanine</td>
</tr>
<tr>
<td>168</td>
<td>lipid fragment (C(_5)H(_9)NPO(_2^+))</td>
</tr>
<tr>
<td>175</td>
<td>arginine, glutamic acid</td>
</tr>
<tr>
<td>177</td>
<td>asparagine</td>
</tr>
<tr>
<td>178</td>
<td>glycine</td>
</tr>
<tr>
<td>179</td>
<td>alanine</td>
</tr>
<tr>
<td>182</td>
<td>lipid fragment (C(_5)H(_9)NPO(_2^+)), tyrosine</td>
</tr>
<tr>
<td>184</td>
<td>lipid fragment (C(_5)H(_9)NPO(_2^+))</td>
</tr>
<tr>
<td>188</td>
<td>tryptophan</td>
</tr>
<tr>
<td>190</td>
<td>lipid fragment (C(_5)H(_9)NPO(_2^+))</td>
</tr>
<tr>
<td>194</td>
<td>lipid fragment (C(_5)H(_9)NPO(_2^+))</td>
</tr>
<tr>
<td>196</td>
<td>lipid fragment (C(_5)H(_9)NPO(_2^+))</td>
</tr>
<tr>
<td>198</td>
<td>lipid fragment (C(_5)H(_9)NPO(_2^+))</td>
</tr>
<tr>
<td>200</td>
<td>glycine</td>
</tr>
<tr>
<td>205</td>
<td>tryptophan</td>
</tr>
<tr>
<td>206</td>
<td>lipid fragment (C(_5)H(_9)NPO(_2Na^+))</td>
</tr>
<tr>
<td>210</td>
<td>lipid fragment (C(_5)H(_9)NPO(_2^+))</td>
</tr>
<tr>
<td>212</td>
<td>lipid fragment (C(_5)H(_9)NPO(_2^+))</td>
</tr>
<tr>
<td>219</td>
<td>aspartic acid, cysteine</td>
</tr>
<tr>
<td>224</td>
<td>lipid fragment (C(_5)H(_9)NPO(_2^+))</td>
</tr>
<tr>
<td>226</td>
<td>lipid fragment (C(_5)H(_9)NPO(_2^+))</td>
</tr>
<tr>
<td>231</td>
<td>proline</td>
</tr>
<tr>
<td>235</td>
<td>valine</td>
</tr>
<tr>
<td>238</td>
<td>lipid fragment (C(_5)H(_9)NPO(_2^+))</td>
</tr>
<tr>
<td>239</td>
<td>threonine</td>
</tr>
<tr>
<td>240</td>
<td>lipid fragment (C(_5)H(_9)NPO(_2^+))</td>
</tr>
<tr>
<td>241</td>
<td>cysteine</td>
</tr>
<tr>
<td>246</td>
<td>lipid fragment (C(_5)H(_9)NPO(_2Na^+) or C(_4)H(_13)NPO(_2Li^+))</td>
</tr>
<tr>
<td>252</td>
<td>lipid fragment (C(_5)H(_9)NPO(_2^+))</td>
</tr>
<tr>
<td>254</td>
<td>lipid fragment (C(_5)H(_9)NPO(_2^+))</td>
</tr>
<tr>
<td></td>
<td>Lipid Fragment (C$<em>{18}$H$</em>{37}$NPO$_5$)</td>
</tr>
<tr>
<td>----</td>
<td>----------------------------------------</td>
</tr>
<tr>
<td>256</td>
<td></td>
</tr>
<tr>
<td>263</td>
<td></td>
</tr>
<tr>
<td>279</td>
<td></td>
</tr>
<tr>
<td>282</td>
<td></td>
</tr>
</tbody>
</table>
Table 4.4. Classifying hematopoietic cell lineage.
The sensitivity, specificity, and class error of differentiation stage identification made with the PLS-DA model constructed using the cell-specific peak set and the complete peak set.

<table>
<thead>
<tr>
<th></th>
<th>Cell-Specific Peak Set</th>
<th>Complete Peak Set</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B Cells</td>
<td>CLPs</td>
</tr>
<tr>
<td>Sensitivity of Identification of Calibration Samples</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Specificity of Identification of Calibration Samples</td>
<td>1.0</td>
<td>0.97</td>
</tr>
<tr>
<td>Sensitivity of Identification of Test Samples</td>
<td>0.93</td>
<td>0.83</td>
</tr>
<tr>
<td>Specificity of Identification of Test Samples</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Class Error of Identification of Calibration Samples</td>
<td>0.02</td>
<td>0.10</td>
</tr>
<tr>
<td>Class Error of Identification of Test Samples</td>
<td>0.03</td>
<td>0.08</td>
</tr>
</tbody>
</table>
Table 4.5. Classifying hematopoietic cell age.
The sensitivity, specificity, and class error of identification of the differentiation stage of HCs harvested from old (calibration set) and young (test set) mice determined for the PLS-DA models constructed using the cell-specific peak set and the complete peak set.

<table>
<thead>
<tr>
<th></th>
<th>Cell-Specific Peak Set</th>
<th>Complete Peak Set</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B Cells</td>
<td>CLPs</td>
</tr>
<tr>
<td>Sensitivity of Identification of Calibration Samples</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Specificity of Identification of Calibration Samples</td>
<td>0.98</td>
<td>0.97</td>
</tr>
<tr>
<td>Sensitivity of Identification of Test Samples</td>
<td>0.65</td>
<td>0.95</td>
</tr>
<tr>
<td>Specificity of Identification of Test Samples</td>
<td>0.97</td>
<td>0.53</td>
</tr>
<tr>
<td>Class Error of Identification of Calibration Samples</td>
<td>0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>Class Error of Identification of Test Samples</td>
<td>0.19</td>
<td>0.26</td>
</tr>
</tbody>
</table>
CHAPTER 5: ADHESIVE SIGNATURES OF HEMATOPOIETIC STEM AND PROGENITOR CELLS AS A NEW SINGLE CELL METRIC

5.1. General chapter overview

Adhesive signatures of individual cells may provide significant information regarding the bioactivity of individual hematopoietic stem cells (HSC) and lineage-restricted hematopoietic progenitors. Our current capacity to continuously monitor the functional output of HSCs in vitro limits progress in understanding how extrinsic cues impact HSC fate decisions. Conventional functional assays of HSCs include repopulation assay, colony-forming unit (CFU) assay, long-term culture-initiating cell (LTC-IC) assay, and cobblestone area-forming cell (CAFC) assay but these require long incubation time and are expensive and labor-intensive (58). Means to dynamically monitor single putative HSCs in situ in a label-free manner would therefore supply new insight regarding extrinsic regulation of HSC fate decision processes (57). Recent evidence suggests that adhesive signatures of individual cells may be used as a predictive marker for changes in cell state in the context of cancer (257). For example, Reticker-Flynn et al. demonstrated that the adhesive profiles of primary lung cancer cells on an array of ECM proteins could be used to predict their metastatic potential with stronger adhesion to combinations of fibronectin, galectin-3, galectin-8, and laminin being correlated with higher metastatic activity, suggesting clinical relevance of adhesive signatures of individual cells. However, such approaches are yet to be applied to cells of hematopoietic origin or to examine processes of stem

---

4 This chapter has been adapted from the following publication and manuscript in preparation:


Choi JS, Wang X, Ha T, Harley BAC, Tension gauge tether system to quantify adhesive forces of primary hematopoietic stem cells. In preparation.
cell lineage specification. Here, we hypothesize that adhesive signatures of HSCs can be used as a marker for predicting changes in their functional output. To test this idea, we first aim to obtain adhesive signatures of individual HSCs using a recently described photonic crystal enhanced microscopy (PCEM) approach (111) then attempt to quantify adhesive forces generated between HSCs and the underlying substrate using a recently described tension gauge tether (TGT) system (166). As a proof-of-concept, in this chapter I describe the use of PCEM to collect adhesive signatures of dental epithelial stem cells during cell attachment, apoptosis, and chemotaxis. I subsequently present preliminary data that TGTs can present an adhesive substrate for HSC cultures.

From the outset I want to acknowledge the highly collaborative nature of a major element of this chapter. Notably, §5.2 (Photonic crystal enhanced microscopy (PCEM)) and §5.3 (Tension gauge tether (TGT) system as adhesive force reporters) would not be possible without the assistance and expertise of Prof. Brian Cunningham and his laboratory as well as Prof. Taekjip Ha and his laboratory at the University of Illinois at Urbana-Champaign. Section §5.2 is modified from a joint manuscript co-authored with Weili Chen, Kenny Long, Dr. Meng Lu, Dr. Vikram Chaudhery, Hojeong Yu, James Polans, and Yue Zhuo (Cunningham Lab). Section §5.3 is modified from a joint manuscript currently in preparation with Xuefeng Wang (Ha Lab). I chose to include the entire technical content of both manuscripts in this chapter as it adds significant perspective to this thesis, but from the outset want to acknowledge the contributions of my co-authors and collaborators in these projects.

5.2. Photonic crystal enhanced microscopy (PCEM)
5.2.1. Abstract

Photonic crystal biosensor surfaces provide a substrate for cell attachment as well as an approach to generate time-resolved images of cell-surface interactions in a label-free, quantitative manner at submicron resolution and without the need for potentially cytotoxic staining agents or temporally-unstable fluorophores. More conventional forms of microscopy (light, fluorescence, electron) do not provide such potential for direct measurement of live cell-surface attachment. The ability to resolve dynamic morphological signatures during cell-matrix engagement may by critical to studies of biological phenomena such as stem cell differentiation, chemotaxis, apoptosis, and metastasis. Here, we demonstrate the application of Photonic Crystal Enhanced Microscopy (PCEM) towards resolving the evolution of cell attachment and morphology of murine dental stem cells during chemotaxis and drug-induced apoptosis. We observe that PCEM provides rich, dynamic information about the evolution of cell-surface attachment profiles over biologically relevant time-scales. Critically, this method retains the ability to monitor cell behavior with spatial resolution sufficient for observing both attachment footprints of filopodial extensions and intracellular attachment strength gradients.

5.2.2. Introduction

Cell membrane interactions with surfaces are fundamental aspects of many in vivo biological phenomena including differentiation, growth, apoptosis, tumor metastasis and injury response (258, 259). Characterizing these processes in the laboratory traditionally involves either fluorescent dyes, fluorescent proteins, histological stains, or fixation. Such approaches are either cytotoxic, or temporally constrained by the effects of fluorescence photobleaching. While these techniques elucidate the mechanics and outcomes of cellular processes, the lack of long-term,
time-course data collection poses a serious compromise to the study of natural cell behavior during processes that occur over extended time scales like cell invasion (260) and chemotaxis (261).

In order to address the challenges inherent in label-based cell imaging techniques, label-free microscopy technologies have been demonstrated as effective tools for measuring an increasingly diverse range of cellular processes (262-265). Label-free microscopy involves a biosensor transducer surface that generates an electrical or optical signal when cells interact with it. Biosensors measure intrinsic cellular properties (such as dielectric permittivity) that can be used to determine the number of cells in contact with the transducer, or to determine the distribution of focal adhesion points. Such transducers may be prepared with surface coatings that either selectively capture specific cell populations through interaction with proteins expressed on their outer membranes or mimic the in vivo microenvironment within tissues.

Due to the fundamental importance of cell-surface interactions, several technologies have sought to quantify and image cell membrane adhesion. Surface Plasmon Resonance imaging (SPRi) (266) is capable of detecting cell attachment to a gold surface by measuring changes in the intensity of front-reflected light at a fixed angle and wavelength, but practical limitations degrade image quality. SPRi requires illumination to pass through cell structures, which introduces changes in reflected light intensity that are not related to cell surface attachment, and the lateral propagation distance of surface plasmons limits spatial resolution (267). Interpretation of SPRi images is complicated by the variability of reflected light intensity introduced by scattering, inhomogeneity of the light source, and nonuniformity of the sensor surface (268), while non-normal light via prism coupling hinders the quality of focus (269).
In this report, we demonstrate photonic crystal enhanced microscopy (PCEM) as a label-free biosensor-based cell attachment imaging approach that quantifies cell-surface interactions with spatial resolution sufficient for monitoring intra-cell attachment distribution, and temporal resolution sufficient for generating time-lapse movies during processes that include chemotaxis, apoptosis, differentiation, and division. Critically, these studies can be performed on extracellular matrix (ECM) protein functionalized substrates, retaining the capacity to define the chemistry of cell-matrix interactions. The system is integrated with an incubator, enabling long-term monitoring of cell attachment over substantial time scales (hours to days) without interruption of the culture environment. The key innovation enabling these capabilities is the use of noncoherent illumination of a photonic crystal biosensor and a spectroscopic scanning system that couples with a microscope objective. Here, we demonstrate label-free time-lapse imaging of the attachment and chemotaxis of dental stem cells using PCEM. Single-cell movement and filopodial extension are easily identifiable, yielding significant potential for the future investigation of numerous cellular processes including tumor cell metastasis and stem cell differentiation. While still an *in vitro* environment, the elimination of cytotoxic fluorophores and reporter molecules allows for the controlled study of complex biological processes over extended time periods.

5.2.3. Materials and Methods

5.2.3.1. Nanoreplica Molding of PC Sensors.

The PC sensors used in this study were fabricated using a low-cost nanoreplica molding manufacturing approach that has been described previously (270). Briefly, a silicon wafer molding template with a negative volume image of the desired PC grating structure (period =
400 nm, depth = 120 nm) was fabricated using deep-UV lithography and reactive ion etching. Liquid UV-curable epoxy was pressed between a glass cover slip (0.17 mm thick) and the silicon wafer, and was subsequently cured to a solid using a high intensity UV lamp. The hardened epoxy preferentially adhered to the glass substrate and was peeled away from the silicon wafer, leaving a replica of the silicon mold. A thin TiO₂ layer (t~60 nm) was deposited via reactive RF sputtering (PVD 75, Kurt Lesker) providing the high-refractive index coating. Figure 1 b presents a scanning electron micrograph of the replica-molded sensor after dielectric coating and shows excellent uniformity across the PC surface. The final grating height is ~ 80 nm, as measured by atomic force microscope, to provide a surface that does not contain deep or abrupt grooves that may influence cellular attachment processes. The PC is designed to resonantly reflect a wavelength of λ ~ 620 nm, with Δλ = 4 nm attributed to aqueous immersion.

5.2.3.2. Cell Culture.

Murine dental pulp stem cells (mHAT9a) were attained from the Harada Lab (271). Stable cultures of both the wild type and CXCR4 deficient mutant were maintained in Dulbecco’s Modified Eagle Medium supplemented with B-27 serum free supplement (Invitrogen), 20 ng/ml bFGF and 20 ng/ml EGF (Peprotech). Deficient mutant cells were not cultured more than fourteen days before use due to passage number-associated mutations. Panc-1 pancreatic carcinoma cells (ATCC) were maintained in DMEM supplemented with 10% FBS and 4mM L-glutamine.
5.2.3.3. Sensor Preparation.

PCs were sonicated for one minute in acetone, followed by cleaning with IPA and water. After thorough drying with N₂, devices were oxygen-plasma treated for 5 minutes to facilitate attachment of a liquid containment gasket. A polydimethylsiloxane (PDMS) gasket with an internal area of 1.2x1.2 mm² was prepared with a thickness of 8mm. After application of the gasket well, the PC surface was hydrated with PBS, and a layer of fibronectin was adsorbed to promote cellular attachment. The sensor was inserted into a custom holder attached to the motorized stage of the microscope, followed by PWV image scanning.

5.2.3.4. Chemotaxis Investigation.

mHAT9a cells were attached to a fibronectin-treated PC biosensor. Two hours after attachment, soaked beads were warmed to 37 °C and placed in the PCEM field of view using the eyepiece of the microscope for targeting. PCEM images were acquired at 20 minute intervals for a total duration of 10 hours.

5.2.4. Results

5.2.4.1. Photonic crystal biosensor surface engineered specifically for cell attachment

Photonic Crystal (PC) biosensors have recently been demonstrated as a highly versatile technology for a variety of label-free assays including high-throughput screening of small molecule-protein interactions, characterization of protein-protein interactions, and measurement of cell attachment modulation by drugs (272-274). A PC is a sub-wavelength grating structure consisting of a periodic arrangement of a low refractive index material coated with a high reflective index layer (Fig. 5.1). When the PC is illuminated with a broadband light source, high
order diffraction modes couple light into and out of the high index layer, destructively interfering with the zeroth-order transmitted light (275). At a particular resonant wavelength and incident angle, complete interference occurs and no light is transmitted, resulting in 100% reflection efficiency. The resonant wavelength is modulated by the addition of biomaterial upon the PC surface, resulting in a shift to a higher wavelength. The electromagnetic standing wave that is generated at the PC surface during resonant light coupling inhibits lateral propagation, thus enabling neighboring regions on the PC surface to display a distinct resonant wavelength that is determined only by the density of biomaterial attached at that precise location. By measuring the resonant peak wavelength value (PWV) on a pixel-by-pixel basis over a PC surface, an image of cell attachment density may be recorded. PWV images of the PC may be gathered by illuminating the structure with collimated white light through the transparent substrate, while the front surface of the PC is immersed in aqueous media.

The advantages of PC-based surfaces for cell attachment imaging are compelling. As a label-free technology, cell attachment to a PC sensor is measured without the use of dyes or stains, so a population of cells can be measured repeatedly without disrupting their culture environment. The detected output signal is highly quantitative, providing measurements that are repeatable between sensors, instruments, and laboratories without photobleaching. PC biosensors are fabricated from inexpensive materials and require only low intensity illumination from beneath the sensor, so no electrical or physical contact between the sensor and the detection system occurs, and illumination does not pass through the cell body, the cell media, or the liquid-air meniscus of a microplate well. The PC biosensor strictly limits lateral propagation of resonantly coupled light, enabling imaging-based detection with resolution sufficient for measuring subtle variations in cell adhesion strength within a single cell, without needing to pre-
tune the sensor to a particular resonant coupling condition, as in SPRi. PC biosensor imaging provides information that is fundamentally different than that provided by an optical microscope, as the sensor responds to local variation in cell attachment strength to the transducer surface. The sensor can be prepared with a variety of surface functionalizations (such as matrix coatings, antibodies, and peptides) and thus can be used as a tool for measuring how cell attachment to surfaces is modulated by drugs, growth factors, or other environmental factors.

5.2.4.2. Hyperspectral imaging microscope detection instrument

A schematic diagram of the PCEM instrument is shown in Fig. 5.2. The system is built upon the body of a standard microscope (Carl Zeiss Axio Observer Z1), but in addition to ordinary bright field imaging, a second illumination path is provided from a fiber-coupled broadband LED (Thorlabs M617F1, 600 < λ < 650 nm). The fiber output is collimated and filtered by a polarizing beamsplitter cube to illuminate the PC with light that is polarized with its electric field vector oriented perpendicular to the grating lines. The polarized beam is focused by a cylindrical lens (f = 200 mm) to form a linear beam at the back focal plane of the objective lens (10x, Zeiss). After passing through the objective lens, the orientation of the line-shaped beam is rotated to illuminate the PC from below at normal incidence. The reflected light is projected, via a side port of the inverted microscope and a zoom lens, onto a narrow slit aperture at the input of an imaging spectrometer. The width of the adjustable slit was set to 30 µm for the work reported here. Using this method, reflected light is collected from a linear region of the PC surface, where the width of the imaged line, 1.2 µm, is determined by the width of the entrance slit of the imaging spectrometer and the magnification power of the objective lens. The system incorporates a grating-based spectrometer (Acton Research) with a 512 × 512 pixel CCD camera
(Photometrics Cascade 512). The line of reflected light, containing the resonant biosensor signal, is diffracted by the grating within the spectrometer (300 lines/mm) to produce a spatially resolved spectrum for each point along the line. Therefore, each pixel across the line is converted to a resonant reflection spectrum, containing a narrow bandwidth (Δλ ~ 4nm) reflectance peak from the PC. The Peak Wavelength Value (PWV) of each peak is determined by fitting the spectrum to a 2nd order polynomial function, and then mathematically determining the maximum wavelength of the function. By fitting all 512 spectra, in a process that takes 20 msec, a line comprised of 512 pixels is generated that represents one line of a PWV image of the PC surface. With an effective magnification of 26x, each pixel in the line represents a ~ 0.6 μm region of the PC surface and 512 such pixels cover a total width of ~300 μm. To generate a two-dimensional PWV image of the PC surface, a motorized stage (Applied Scientific Instruments, MS2000) translates the sensor along the axis perpendicular to the imaged line in increments of 0.6 μm/step. Using this technique, a series of lines are assembled into an image at a rate of 0.1 sec/line and the same area on the PC surface can be scanned repeatedly. Each image is comprised of 512 by n pixels, where n can be selected during each scan session, and each pixel represents a 0.6 x 0.6 μm region of the PC surface. A biosensor experiment involves measuring shifts in PWV. A baseline PWV image is gathered before the introduction of cells, when the PC is uniformly covered by cell media, which is aligned and mathematically subtracted from subsequent PWV images gathered during and after cell attachment.
5.2.4.3. Characterization of the PC sensitivity and resonant wavelength stability under cell culture conditions

In preparation for cell attachment demonstrations, the ability of the PCEM to measure shifts in the bulk refractive index of the cell media was established. First, exposing the entire PC surface to distilled water (n = 1.333) and subsequently exposing the same sensor to isopropyl alcohol (n = 1.377), we confirmed that an individual pixel within a PCEM image demonstrates high reflection efficiency and narrow resonant reflection bandwidth. The PWV shift measured from a single pixel exposed to both media yielded a bulk refractive index sensitivity of $\Delta \lambda / \Delta n = 102 \text{ nm/RIU}$.

A series of PWV images were gathered over a 12 hour period to demonstrate PWV stability with the PC exposed to cell media at the elevated temperature (T = 37 °C) and 5% CO$_2$ environment used for biological studies. Ham’s F12 media (Invitrogen) was placed in a PDMS well attached to the PC surface. The average PWV shifted by only 0.12 nm over 12 hours, and the standard deviation of PWV within an image of a PC uniformly exposed to cell media was 0.09 nm.

5.2.4.4. Label free imaging of intracellular attachment and morphological changes

The most commonly used method for identification of differentiating stem cells is the labor- and time-intensive methylcellulose assay, which only reveals the identity of the colonies weeks after commitment occurs (276-278). Furthermore, this approach requires cells to be resuspended in liquid phase, thereby altering many of the environmental cues that yielded the observed differential development. Using PCEM, it is possible to capture the dynamics of cell morphology and cell-matrix interactions during complex processes such as stem cell
differentiation under real-time conditions (with less than 60 seconds between subsequent images). Such a tool would be critical for examining the potential of cell attachment signatures as a proxy for stem cell lineage commitment, particularly considering such analyses could be performed without disturbing the extracellular environment.

As cells attach and spread, positive PWV shifts are observed due to an increase in the concentration of cellular material within the evanescent field region of the PC. A PWV image for human pancreatic cancer cells (Panc-1) is compared to a brightfield image of the same cells in Fig. 5.3. Morphological profiles are consistent with healthy, attachment-dependent cells. Representative spectra are shown from inside and outside the cell region, demonstrating a definitive whole-spectrum shift of the characteristic resonant peak. Clearly visible boundaries of ~0.5 nm PWV shifts demonstrate the ability of PCEM to provide information about the geometry of attachment, which has been shown to have significant implications for both the classification of differentiating stem cells (279), and the metastatic potential of tumor cells (280). In addition, sub-cellular variation of PWV is indicative not only of the presence of cellular adhesion, but also modulation in the strength of attachment. For example, cell ‘B’ in Fig. 5.3 shows a gradient in cell attachment strength from left to right. A region of greater PWV shift along the leading edge suggests the formation of lamellipodia, indicating a higher concentration of intracellular matter. Such behavior is consistent with the formation of actin bundles at sites of focal adhesion.

5.2.4.5. Label free imaging of stem cell attachment and drug-induced apoptosis

Next, murine dental stem cells (mHAT9a) were cultured and allowed to attach to a PC surface prepared with fibronectin over a period of two hours (Fig. 5.4). From the series of PWV
images gathered at 3 minute intervals, initial attachment times can be identified within the 3 minute period of image acquisition. Cells are observed attaching to the treated surface, with initial attachment characterized by small, round areas of PWV shift, consistent with spherical cells exiting suspension. As time progresses, average cell diameter increases, and membrane boundaries become more irregular as cellular processes begin to extend from cell bodies. Many cells maintain highest shifts at their periphery, which is consistent with the high concentration of cytoskeletal protein necessary for boundary maintenance and lamellar extension (281). Random locomotion is observable, which reveals that cellular detachment results in a full recovery of initial PWV values when a cell moves to a new location. We observe no preference for the cells to extend themselves or to move along the direction of the PC grating.

Cellular apoptosis and detachment were also studied. Using another fibronectin-treated PC biosensor, mHAT9a cells were allowed to attach to the sensor surface for 3 hours. A final concentration of 2 μM staurosporine, shown to induce apoptosis via protein kinase inhibition (282, 283), was added to the cell chamber and mixed for 15 seconds. Cells were imaged every 20 minutes for 18 hours (Fig. 5.4). Initial cells appear healthy, with various filopodia extending radially from cell bodies. As time progresses, the footprint of the cell bodies decreases, and several of the cells appear to detach completely. Other cells appear to undergo apoptosis prior to detachment, leaving behind remnants of cell membrane, which still produce a detectable PWV shift. The breakdown and modification of cell-cell and cell-ECM interactions is of great importance to answering questions about the progression of cancer cell detachment and metastasis from primary tumor sites. PCEM is unique in the fact that the biosensor response is a direct quantification and 2D localization of attachment at the single cell level, as opposed to indirect methods of staining for actin bundle formation or even ensemble averaging of bulk
dielectric properties. This direct observation available over a time scale of hours to days provides a natural tool for the future study of cancer cell detachment and metastasis.

5.2.4.6. Label free imaging of stem cell chemotaxis

We next sought to validate the use of PCEM imaging in examining cell-mediated chemotaxis. The importance of stromal cell-derived factor-1 (SDF-1α) in the directed chemotaxis of differentiating cells is well-known for a myriad of situations including hypoxic ocular neovascularization, capillary formation and adipocyte differentiation in human adipose tissue, and bone regeneration in traumatic brain injury (284-286). More recently, SDF-1α and its effect in attracting CXCR4 receptor positive cells have been investigated in dental healing and regeneration. However, current mechanisms for studying the recruitment of dental pulp stem cells have been based on fixing and staining cells (287, 288). As the observed migration occurs on the order of days, label-based assays are not feasible for extended time course studies. PCEM provides an opportunity to monitor such events as they occur.

We examined mHAT9a chemotaxis in response to beads soaked in SDF-1α, a chemoattractant to which the receptor CXCR4 is sensitive (289) (Fig. 5.5). After a bead was placed on the sensor surface, attached cells were observed to move in the direction of the eluting bead. Probing lamellipodia extend in multiple directions around the cell, but only projections formed in the direction of the bead are maintained by the migrating cells. Attachment in the trailing edge of the cell decreases over time as the cell bodies proceed in the direction of chemotaxis, resulting in a return of the sensor to its native state. The experiment was repeated with a mHAT cell line with a constitutive knockout of the CXCR4 coding gene, and no directional movement was observed. This suggests that the observed cellular movement was
indeed due to chemotaxis, as opposed to nonspecific locomotion. Critically, we do not observe
preferential movement or extension of cell processes in the direction of the PC grating lines. To
our knowledge, this represents the first label-free time-lapse imaging of the attachment
localization of living cells during chemotaxis.

It has been shown that SDF-1α/CXCR4 mediated recruitment of dental pulp stem cells is
likely an important inflammatory response and underlying promoter of reparative dentin
formation(290). Further investigation of the SDF-1α/CXCR4 pathway with the PCEM
technology could provide a valuable investigation of morphological changes induced by the
inflammatory response of dental pulp stem cells to dental damage.

5.2.5. Discussion and Conclusions

PCEM represents a new imaging modality that can be easily integrated with a
conventional optical microscope to enable quantified, near real-time, high resolution imaging of
cell-surface interactions. PCEM provides information that is not available from ordinary bright
field imaging or phase contrast imaging, but instead, by virtue of the surface-confined resonant
electric field of the PC, enables high contrast imaging of the interaction strength of cells with a
surface. The approach utilizes low power illumination from a visible wavelength LED from
below, using a PC sensor structure that can be incorporated into standard coverslips (as
demonstrated here), microscope slides, or microtiter plates that are typically used for cell
research. PCEM clearly demonstrates that cell-surface attachment strength is not uniformly
distributed within a cell or static as a function of time, but instead contains rich dynamic
information that includes the rate of cell boundary extension, the size of a cell “footprint” on a
surface, and the effect of the extracellular environment (including chemotactic gradients) on cell attachment.

The cell imaging experiments used to demonstrate PCEM were selected to show that the spatial resolution of the approach is sufficient for clearly observing features such as spatial gradients in cell-surface attachment and the extension of fine-structured filopodia, attributes that are typically observed only using dyes or stains. As a label-free detection approach, PCEM enables continuous monitoring of these phenomena over extended time periods that are compatible with the biological time scales of chemotaxis, apoptosis, differentiation, and proliferation. This work demonstrates, to our knowledge, the first time-lapse movies of cell-surface interaction monitoring at these time scales.

There is an increasing awareness of the importance of cellular adhesion and the mechanical microenvironment of cells on their behavior, yet directly measuring these attributes in a non-invasive fashion has proved difficult. PCEM provides a novel, robust methodology for the investigation of these attributes in a controlled environment without chemical alteration. The relationship between mechanical microenvironmental cues and cancer cell behavior has been demonstrated, contributing significantly to tissue dysplasia and metastatic detachment(291). With PCEM, it will be possible to investigate important components within the progression of tumor-development, such as the recruitment and movement of neutrophils to the cancer microenvironment. Neutrophil polarization and chemotaxis represents a challenging process to study as it presents a complex and dynamic set of cellular-ECM interactions. Near real-time imaging would allow for rapid improvement in our understanding of this and the other biological applications discussed herein, providing dynamic attachment information that is not currently available.
5.3. Tension gauge tether (TGT) system as adhesive force reporters

5.3.1. Abstract

Cells attach and adhere to substrates with varying degrees of forces. Recently, a tension gauge tether (TGT) was developed as a single-molecule based approach to quantify adhesive forces generated during cell-matrix engagement (166). TGT is a platform that enables the measurements of single-molecule forces required to engage substrates to receive mechanical signals. The tether consists of a ligand (tether) covalently attached to DNA base pairs whose rupture force can be tuned based on the position of the tether. The rupture force corresponds to the maximum tension that the TGT can withstand when receptors on the cell surface engage its ligands. Relative position of attachment of one side of the TGT to the substrate of interest and the other single DNA strand to the adhesive ligand enable facile modification of the failure strength of the TGT. The TGT system hence allows the quantification of single-molecule forces of adhesion to specific ligands. Using this system, we quantified adhesive forces of primary hematopoietic stem cells (HSC) engaging RGDfK- or YIGSR-coated glass substrates.

5.3.2. Materials and Methods

5.3.2.1. Substrate preparation

Peptide-coated glass bottom 96-well plates (In Vitro Scientific, Sunnyvale, CA) were prepared via aminosilanization of glass. In brief, glass bottom well plates were cleaned and rendered hydrophilic by exposing to oxygen plasma for 5 min. The glass surface was then aminosilanized and annealed at 80 °C for 4 hours. The glass surface was subsequently reacted with mPEG-SVA and biotin-mPEG-SVA (10:1 ratio; MW 5,000; Laysan Bio, Arab, AL). It was incubated with NeutrAvidin (Fisher Scientific, Hampton, NH) and biotinylated RGDfK (Peptides
International, Louisville, KY) or YIGSR (laminin pentapeptide; AnaSpec, Fremont, CA) to immobilize RGDfK or YIGSR on the surface. Alternatively, biotin-TGT-RGDfK produced as previously described (166) was immobilized to create a TGT platform (Fig. 5.6).

5.3.2.2. HSC isolation and culture conditions

HSCs were isolated from the femurs and tibias of 4 – 8-week-old C47Bl\6 mice (The Jackson Laboratory, Bar Harbor, ME). Briefly, mice were euthanized with carbon dioxide in compliance with the University of Illinois Institutional Animal Care and Use Committee (IACUC) guidelines. Their femurs and tibias were then collected, crushed, and filtered through a 40-µm cell strainer (BD Falcon, Franklin Lakes, NJ) to yield a whole bone marrow cell suspension in PBS supplemented with 2% FBS. Red blood cells were lysed using ACK lysis buffer (Invitrogen, Carlsbad, CA) and the resulting suspension was incubated with Fc receptor blocking antibody (CD16/CD32) to prevent nonspecific antibody binding. The cells were then incubated with a cocktail of FITC-conjugated lineage (Lin) antibodies (CD5, CD45R (B220), CD11b, Anti-Gr-1 (Ly-6G/C), 7-4, Ter-119), PE-conjugated Sca-1, and APC-conjugated c-Kit to isolate Lin\-Sca-1\+c-Kit\+ (LSK) via fluorescence-activated cell sorting (FACS) using BD FACSARia™ cell sorter. Dead cells were excluded with propidium iodide (PI) staining and sorting consistently yielded 4E4 – 6E4 HSPCs per mouse All antibodies purchased from eBiosciences, San Diego, CA.

Isolated LSK cells were cultured on peptide-coated glass substrates in serum-free StemSpan™ HSC expansion medium (Stem Cell Technologies, Vancouver, Canada) supplemented with 10% FBS (cytokine-free) or with 100 ng/mL of SCF, Flt3L, and TPO (serum-free) (Peprotech, Oak Park, CA).
5.3.2.3. Analysis via colony-forming unit (CFU) assay

Cultured HSCs were lifted with 0.05% trypsin and resuspended in methylcellulose medium supplemented with murine cytokines (MethoCult® GF M3434; Stem Cell Technologies, Vancouver, Canada) according to the manufacturer’s protocol. In brief, the methylcellulose medium containing the cells was dispensed to 35mm culture dishes using a 3cc syringe. Samples were prepared in triplicates or quadruplets and incubated for 11-14 days at 37° C and 5% CO2 at which point the colonies that arose with more than 30 cells were enumerated with a light microscope (DMI4000, Leica Microsystems, Germany). CFU-GEMM, CFU-GM, CFU-M, CFU-G, CFU/BFU-E, and CFU-Mk colonies were enumerated.

5.3.3. Preliminary Results and Ongoing Work

RGDfK-coated glass substrates were prepared using TGTs with a rupture force of 12 pN or 54 pN to create a TGT platform (Fig. 5.6). Previously, it was found that a threshold force of 43 pN is required to activate αvβ3-mediated binding to RGDfK in a number of cell lines including CHO-K1, HeLaAa, and NIH-3T3 cells (166). In contrast, HSCs could adhere with forces smaller than 12 pN (Fig. 5.7) within 3-hours of ex vivo culture to receive mechanical signals that induced changes in colony-forming potential (Fig. 5.8). Here, TGTs were decorated with Cy3 to enable fluorescent visualization. As seen in Fig. 5.8, cells adhering with forces greater than the rupture force of the TGTs tore off the TGTs to appear fluorescent. Cells adhering with forces smaller than the rupture force appear darker than the background although it is difficult to see due to the bright background signal.
Notably, αvβ3-mediated interactions with RGDfK-coated glass substrates induced lineage specification as early as within 3-hours of *ex vivo* culture. Once such lineage commitment decisions were made, they persisted throughout the 24-hour culture (Fig. 5.8). Our results suggest that HSC fate decisions are sensitive to even very subtle changes in matrix cues. Ongoing work is investigating adhesion of HSCs to other peptides including YIGSR (laminin pentapeptide) to further explore the effect of specific integrin/receptor activation in combination with single-molecule adhesion forces.
5.3. Figures

Figure 5.1. Schematic diagram of the photonic crystal (PC) biosensor. 
(A) Schematic diagram of the photonic crystal (PC) biosensor. A PC sensor is comprised of a replica molded polymer grating overcoated with a high refractive index thin film of TiO$_2$. Inset: photo of a PC fabricated upon a glass cover slip. (B) Scanning electron micrograph of the PC surface.
Figure 5.2. Instrument schematic of the PCEM.
Illumination from a fiber-coupled LED is collimated and passed through a polarizing beamsplitter (PBS) to create a pure electric field polarization perpendicular to the PC grating. A cylindrical lens focuses the light to a line at the back focal plane of the objective. The PC resonantly reflects only a narrow band of wavelengths, which are collected through the entrance slit of an imaging spectrometer.
Figure 5.3. Imaging of Panc-1 cells.
(a) Bright field and (b) PWV imaging of Panc-1 cells attached to the PC surface. Cells were seeded onto a fibronectin-coated sensor and allowed to incubate for 2 hours before imaging. Lamellipodial extensions are visible, especially from cell 2, demonstrating the ability of PCEM to resolve regional differences in single-cell attachment. Darker shading indicates regions of higher protein concentration, and is present in regions near the boundary of lamellipodia formation, consistent with the creation of actin bundles. (c) Representative regions of cellular attachment. Selected areas of the PWV image from beneath a cell show the PWV shift of a typical Panc-1 cell is 1.0 nm, and consistent throughout the entire spectrum at those locations.
Figure 5.4. Imaging of attachment of mHAT9a cells.
(a) Time lapse PWV images of cellular attachment of mHAT9a cells. Cells were seeded at 20,000 cells per ml on a fibronectin-coated sensor surface. After 3 minutes, regions initial cell attachment appear as small, round regions, consistent with spheroid, trypsinized cells coming out of suspension and attaching to a surface. As time progresses, both the size of the cells and intensity of the PWV shift induced by them increases, indicating a higher localization of cellular material at the sensor surface, which can be expected during cell spreading. Finally, once cells are sufficiently attached, cellular processes can be observed sensing the cells' microenvironment in all directions. The outer irregular boundaries of the cells have a relatively low PWV, consistent with thin, exploratory filopodia, accompanied by a more heavily attached region slightly immediately adjacent in the cell interior, likely a result of actin bundle formation. (b) Time lapse PWV images of mHAT9a apoptosis and detachment. Cells were seeded at 8000 cells per ml onto a fibronectin-coated sensor surface. Cells that detach can be observed by the gradual retraction of filopodia and overall cell rounding before the PWV shift disappears entirely. Some cells appear to undergo apoptosis while still attached, leaving remnants of cell membranes and protein on the sensor surface. ΔPWV data was attained via background subtraction from an initial image taken before cell attachment ($t=0$).
Figure 5.5. Imaging of chemotaxis of mHAT9a cells.
(a) Time lapse PWV images of chemotaxis of mHAT9a cells. Cells were deposited on the sensor surface at a concentration of 8000 cells per ml and allowed to attach for 2 hours before imaging. An agarose bead was placed at a location approximately 100 microns above the top of the image, and PWV images were collected every 20 minutes after the bead was placed. Cell movement direction is indicated with an arrow in the leftmost frame. (b) CXCR4 knockout cells exhibit non-directional movement on the sensor surface. Similarly prepared, CXCR4 mutants do not show directional movement toward the bead, demonstrating that the previously observed directional locomotion was due to chemotaxis.
Figure 5.6. TGT system.

a, Rupture force of TGTs can be tuned by adjusting the position of the tether (ligand) (166). b, Schematic of the TGT-functionalized glass substrates with. In our system, TGTs with a rupture force of 12 pN or 54 pN were used. c, Glass modification was visualized by adding FITC-avidin in place of NeutrAvidin. Scale bar: 100 μm.

Image modified from:  Wang, Ha (2013) (166)
Figure 5.7. Adhesion of HSCs to TGT-RGDfK.
HSCs could adhere to TGT-RGDfK-coated glass substrates with a rupture force of smaller than 12 pN within 3-hours of ex vivo culture. a, 12 pN and b, 52 pN TGTs. Scale bar: 100 μm.
Figure 5.8. CFU colonies from HSCs cultured on RGDfK-coated glass.
HSCs were cultured on RGDfK-coated glass for up to 24 hours. Lineage specification occurred within 3-hours of culture. a, Overall CFU colony results. b, CFU-GEMM, CFU-M, and CFU-E are shown (*:p < 0.05)
CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

6.1. Conclusions

Hematopoietic stem cells (HSC) reside in the bone marrow within specialized microenvironments known as niches, comprised of various cellular and extracellular components including niche cells, extracellular matrix (ECM), and immobilized or soluble signaling molecules that provide extrinsic cues to regulate HSC fate decisions: quiescence, self-renewal, differentiation, mobilization, migration, retention, and apoptosis. Variations in structure and composition across the proposed niche microenvironments within the bone marrow are believed to present a complex landscape of microenvironmental signals that may selectively contribute to hematopoiesis by favoring certain HSC fate decisions over others. However, teasing apart the effect of niche-inspired biophysical cues on HSC fate decision processes has remains a challenge due to the rarity of bona fide HSCs, our lack of technical ability to expand or differentiate HSCs freely in vitro, and unavailability of single cell metrics to continuously analyze the functional output of HSCs in situ. Consequently, despite extensive research efforts that have concentrated primarily on resolving the biochemical elements of the HSC niche, no biophysical explanation existed for the niche-mediated regulation of HSC proliferation and lineage specification.

Here, we provide critical evidence supporting the presence of a direct link between the biophysical environment of the marrow and early HSC fate decisions using marrow-inspired ECM protein-coated polyacrylamide substrates as an engineered matrix platform with defined stiffness and ligand presentation (Chapter 1 and 2). Specifically, our CFU data highlights the importance of integrin activation and actomyosin contraction in the dynamic regulation of early HSC fate, particularly regarding quiescence/self-renewal vs. lineage specification. Notably, microenvironmental conditions resembling the endosteal niche (high fibronectin content, stiffer)
supported primitive hematopoietic stem and progenitor cells while those resembling the perivascular niche (high laminin content) encouraged differentiation activity, particularly towards erythroid lineage via myosin II-dependent contractility and α5β1-mediated adhesion. Our report therefore established the utility and relevance for using context-driven matrix cues as design parameters for building an \textit{in vitro} platform for the expansion or selective differentiation of HSCs.

Because \textit{in vitro} culture platforms would require continuous monitoring of any changes to HSC fate decisions \textit{in situ}, we evaluated the feasibility of single cell analysis methods (atomic force microscopy (AFM), time-of-flight secondary ion mass spectroscopy (TOF-SIMS), Raman spectroscopy) as new functional metrics of HSCs (Chapter 4). Notably, AFM was found to not be suitable for the analysis of hematopoietic stem and progenitor cells due to large lateral instability during force indentations although it was useful for the mechanical characterization of soft hydrogels. However, TOF-SIMS data revealed compositional differences in the cell membranes of primary hematopoietic cells but this method was limited to the analysis of fixed samples. Finally, preliminary results with Raman spectroscopy offered promising results for Raman approaches to generate time-lapse information regarding single cells cultured on engineered biomaterial substrates.

As recent evidence suggests that adhesive signatures of individual cells may be used as a predictive marker for changes in cellular state, we also considered the use of adhesive signatures of individual cells as a marker for predicting changes in the functional output of HSCs and lineage-restricted progenitors. As a proof-of-concept, we used photonic crystal enhanced microscopy PCEM as a platform for collecting adhesive signatures of individual dental epithelial stem cells during chemotaxis. Finally, given the observed significance of the biophysical
properties of biomaterial substrates on HSC fate, we explored the potential for using a recently described tension gauge tether (TGT) approach to resolve adhesive forces of HSCs.

6.2. Future Directions

Our long-term goal is to build an in vitro culture platform with the capacity to analyze single cells in situ for the expansion or selective differentiation of HSCs for clinical and research applications. For this, we have to continue our effort to better understand how context-driven matrix cues mediate intrinsic mechanosensing processes in hematopoietic stem and progenitor cells. As the regulatory potential of marrow niche cells has also been described, moving forward it will be critical to identify approaches to integrate marrow-derived niche cells as a means to sequentially build increasingly complex microenvironments. We also need to establish single cell analysis methods particularly Raman spectroscopy as new functional metrics of live HSCs at the single cell level. Assessing the potential for using adhesive signatures as a measure of changes in the functional output of HSCs would be another milestone. As some or all of the elements in these endeavors are uncovered, future projects would be expected to develop novel biomaterials system to engineer HSC fate decisions in vitro.
APPENDIX A: GEL FABRICATION AND CHARACTERIZATION PROTOCOLS

A.1. Collagen Hydrogel Fabrication Protocol

REFERENCES: (125, 126)

SUPPLIES
Type I collagen from rat tail (BD Biosciences, Bedford, MA); Respective cell culture medium; 1M HEPES buffer; 0.4 M NaOH; 10-mm or 14-mm glass bottom dish (MatTek Corp., Ashland, MA)

EQUIPMENT
Biological safety cabinet; pH meter; 37 °C incubator

PROCEDURE
NOTE: Pre-cool all reagents required for collagen gel fabrication to 4 °C before use to prevent any premature collagen gelation upon mixing.

1. Dilute collagen stock solution (8.25 mg/mL to 9.33 mg/mL) with the respective cell culture medium to bring the final concentration to 1.45 or 2.9 mg/mL, as desired. Make 1-mL or 2-mL working volume of the collagen mixture.

2. Add 50 µL of 1 M HEPES buffer and appropriate amounts of 0.4 M NaOH to bring the pH to 7.4 according to previously established calibration curves. An example of such curve is shown below. Mix well.

![Figure A.1. Calibration curve showing the amount of 0.4 M NaOH that needs to be added to the collagen mixture (2-mL working volume) in order to bring the pH to 7.4.](image)

3. Place the collagen mixture onto a 10-mm or 14-mm glass bottom dish.

4. Incubate for at least 2 hours in a humidified 37 °C incubator for complete polymerization.
A.2. Collagen Hydrogel Cell Encapsulation Protocol

REFERENCES: (125, 126)

SUPPLIES
Type I collagen from rat tail (BD Biosciences, Bedford, MA); Respective cell culture medium containing a desired number of cells; 1M HEPES buffer; 0.4 M NaOH; 10-mm or 14-mm glass bottom dish (MatTek Corp., Ashland, MA)

EQUIPMENT
Biological safety cabinet; pH meter; 37 °C incubator

PROCEDURE
NOTE: Pre-cool all reagents required for collagen gel fabrication to 4 °C before use to prevent any premature collagen gelation upon mixing.

1. Dilute collagen stock solution (8.25 mg/mL to 9.33 mg/mL) with the respective cell culture medium containing a desired number of cells to bring the final concentration to 1.45 or 2.9 mg/mL, as desired. Make 1-mL or 2-mL working volume of the collagen mixture.

2. Add 50 µL of 1 M HEPES buffer and appropriate amounts of 0.4 M NaOH to bring the pH to 7.4 according to previously established calibration curves. An example of such curve is shown in Figure A.1. (Appendix A.1.)

3. Place the collagen mixture onto a 10-mm or 14-mm glass bottom dish.

4. Place it in a humidified 37 °C incubator. Collagen gel will polymerize and give 3D structural support to the encapsulated cells. In addition, the culture of embedded cells is initiated at this point.
A.3. SEM Imaging of Collagen Hydrogel Protocol

SUPPLIES
Type I collagen from rat tail (BD Biosciences, Bedford, MA); Respective cell culture medium; 1M HEPES buffer; 0.4 M NaOH; Liquid nitrogen

EQUIPMENT
SEM (JEOL JSM-6060LV Low Vacuum SEM, JEOL Ltd., Tokyo, Japan); Freeze dryer (VirTis Genesis, Gardiner, NY)

PROCEDURE
1. Make collagen gel solution to be analyzed according to the procedures detailed in Appendix A.1.
2. Pour liquid nitrogen directly on top of the fabricated collagen hydrogel to snap freeze it.
3. Quickly move the gel to a pre-cooled (4 °C) freeze-dryer and freeze dry it for 18 hours.
4. Take SEM images of the prepared gel at 200x magnification, 20 kV, and 10 Pa.

REFERENCES: (125, 126)

SUPPLIES
Type I collagen from rat tail (BD Biosciences, Bedford, MA); Respective cell culture medium; 1M HEPES buffer; 0.4 M NaOH

EQUIPMENT
Bohlin C-VOR rheometer (Malvern Instruments, Westborough, MA) with a built-in temperature bath for temperature control

PROCEDURE
NOTE: Pre-cool all reagents required for collagen gel fabrication to 4 °C before use to prevent any premature collagen gelation upon mixing.

1. Prepare collagen gel solution according to the procedures detailed in Appendix A.1. Make at least 350 µL to analyze.

2. Zero the rheometer (i.e. gap height = 150 µL). Attach a 4° 20-mm cone geometry.

3. Cool the stage to 25 °C.

4. Place 350 µL of the collagen solution onto the center of the pre-cooled stage.

5. Lower the 4° 20-mm cone geometry down to press the loaded collagen solution. Excess solution around the cone can be trimmed using Kimwipes.

6. Place two small sponges soaked with water on the inside of the solvent trap. Carefully place the solvent trap on top of the loaded collagen gel sample and the cone in order to prevent evaporation during analysis.

7. Open the Bohlin software and adjust settings on the software. Choose oscillatory mode with 1 Hz and 1% strain rate. Also set the software so that rheological measurements will be taken for three consecutive minutes every 20-min intervals.

8. Start the measurement. Raise the temperature of the stage to be 37 °C as soon as the test is started. Temperature will be kept at 37 °C throughout the remainder of the measurement.

9. Software records moduli data over a 12-hr test period.
A.5. PA Gel Fabrication Protocol

REFERENCES: (122, 124)

SUPPLIES
35 mm glass bottom culture dishes (MatTek Cat # P35G-0-14-C; 14mm microwell)
Circular glass coverslips (Fisher Cat # 12-545-80; 12mm in dia.)
0.1N NaOH
3-aminopropyltrimethoxysilane (keep from moisture)
30 v/v% acrylamide stock solution (light sensitive); from acrylamide powder (Sigma)
   3.39g acrylamide in 10 mL solution; density = 1.13 g/cm³
0.5% glutaraldehyde in PBS (store at 4C)
   0.625 mL of 8% stock solution + 9.375 mL of 1x PBS = 10 mL 0.5% solution
   3.125 mL of 8% stock solution + 46.875 mL of 1x PBS = 50 mL 0.5% solution
10% APS (make fresh solution every month; keep from light)
   1.9g APS in 10mL solution; density = 1.9 g/cm³
Tetramethylethylenediamine (TEMED)
Acryl/bis-acryl mixture (light sensitive); Fisher Cat# AC33020-1000
50 mM Hepes (pH 8.5)
dichlorodimethylsilane (DCDMS)
DI water

EQUIPMENT
37° incubator; Vortex

PROCEDURE
Day 1:
1. Pipette 200-400 uL of 0.1N NaOH onto each glass substrate of a glass bottom culture dish. Sufficient NaOH should be added to cover the entire glass surface.

2. Leave it overnight to dry. Evenly distributed crystals should form on the surface by Day 2.

Day 2:
1. Pipette 100 uL of 3-aminopropyltrimethoxysilane (silanizing agent) onto the NaOH-crystallized glass substrate. Let it dissolve the previously formed NaOH crystals completely.

2. Wait 5 minutes then wash 2x with deionized water.

3. Pipette 200 uL of 0.5% glutaraldehyde in PBS onto the glass substrate. Let it sit for 30 minutes then wash 2x with deionized water.

4. Remove excess liquid with Kimwipes and leave the dish to air dry completely.

5. Make a PA mixture.
   Take 994.5 uL of acryl/bis mixture solution of a desired concentration.
Add 5uL of 10% APS (1/200 vol.), 0.5uL of TEMED (1/2,000 vol.) into the mixture. Vortex thoroughly.

**Table A.1.** Acrylamide/bis-acrylamide concentrations required to make PA gels with a desired elastic modulus.

<table>
<thead>
<tr>
<th>Acryl % (v/v)</th>
<th>Bis % (v/v)</th>
<th>Elastic modulus</th>
<th>Dilution</th>
</tr>
</thead>
</table>
| 4             | 0.03        | 0.71 kPa        | Acryl/bis stock = 0.15 mL  
30% acryl = 1.143 mL  
DI water = 8.707 mL |
| 5             | 0.1         | 3.5 kPa         | Acryl/bis stock = 0.5 mL  
30% acryl = 1.033 mL  
DI water = 8.467 mL |
| 5             | 0.26        | 8.75 kPa        | Acryl/bis stock = 1.3 mL  
30% acryl = 0.020 mL  
DI water = 8.680 mL |
| 10            | 0.3         | ~35-44 kPa      | Acryl/bis stock = 1.5 mL  
30% acryl = 1.433 mL  
DI water = 7.067 mL |

6. Quickly place **15 uL of acrylamide/bis-acrylamide** mixture onto each glass substrate.  
10 μL ~ 70 um thickness (ref: N. Wang Lab)  
15 μL ~ 75 um thickness (ref: N. Wang Lab)

7. Place a circular coverslip on top of the placed solution then flip over the dish. Keep the dish in the incubator (37C) for 30 minutes. This will ensure that flat gel surface. For 196 kPa PA gels, incubate at room temperature for 10-15 min because the reaction is fast and very exothermic. Alternatively, place a pre-chlorosilanized coverslip on top of the polymer solution and incubate for 30 minutes.

**Chlorosilanization of glass coverslips:**

1. Soak the coverslips in DCDMS for 5 minutes.  
2. Remove excess DCDMS with KimWipes.  
3. Rinse coverslips with DI water for 1 minute.

8. Flip over the dish and add DI water to cover the gel. Just add DI water to cover the gel if chlorosilanized coverslip was used.

9. Carefully remove the circular coverslip with a razor blade.

10. To remove unpolymerized acrylamide, rinse twice in DI water, each time for 5 minutes.

11. Store the gel hydrated in DI water at 4C until needed.
A.6. PA Gel Surface Functionalization Protocol

REFERENCES: (122, 124)

SUPPLIES
- PA gel
- Sulfo-SANPAH (Fisher Scientific, Pittsburgh, PA)
- Type I collagen from rat tail (BD Biosciences, Bedford, MA)
- 50 mM Hepes (pH 8.5)
- DI water

EQUIPMENT
- UV lamp (365 nm; UVP B-100 High Intensity UV lamp); Vortex

PROCEDURE

1. Take a PA gel and remove liquid with Kimwipes. Careful not to touch the gel itself.

2. Pipette **100 uL of SANPAH** onto each gel surface.
   - Sulfo-SANPAH (keep from light) = 50 mg
   - DMSO = 500 uL
   - 50mM HEPES = 50 mL
   *Add DMSO first, then slowly add the HEPES to the sulfo-SANPAH while vortexing.

3. Expose the dish to the UV light (365 nm) at a distance of 2-3 inches for 6 minutes. Turn on the UV lamp and leave it on for 8 minutes. It takes roughly 30s-1.5min for the lamp to fully turn on.

4. Wash 2x with 50mM HEPES.

5. Repeat #2 – 4.

6. Pipette **200 uL of the desired protein solution** at a desired concentration. Protein solution is made by diluting the stock protein solution in 50 mM HEPES at pH 8.5.

7. Let it activate overnight (> 18 h) at 4C.

8. Remove excess protein solution and rinse 2x with DI water.

9. Prior to seeding cells, sterilize the gel by exposing it to UV for 30 minutes in a sterile hood.

10. Store the gels at 4C until needed. However, using the coated gels right away is recommended. DO NOT store gels for more than 2 weeks.
A.7. AFM force measurement protocol

REFERENCES: (292, 293)

SUPPLIES
- PA gel (collagen-coated and uncoated) samples prepared on microscope slides
- Blank glass microscope slide
- DNP-S10 silicon nitride AFM probe (Bruker Corp., Camarillo, CA)
- DI water; Hydrophobic pen

EQUIPMENT
- MFP-3D AFM (Asylum Research, Santa Barbara, CA)

PROCEDURE

Get ready:

1. Turn on Igor software. Select ‘contact mode’.
2. Turn Laser on.
3. Turn light source on.

Figure A.2. A top-down view of the AFM head. Photo-Detector knob adjusts deflection. LDY and LDX knobs control the laser position in y- and x-directions, respectively. Camera control knobs are located near the LDY knob. The big wheel at the front end lifts the head up or down.

NOTE: The head is very heavy and needs to be handled with caution. Also pay attention to the cables attached to the head because it is easy to snap off the cables.
1. Cantilever Calibration

NOTE: Always make sure the head is level! The bubble of the bubble top should be at the center, slightly to the front.

NOTE: RED = Extension; BLUE = Retraction

![Diagram showing force-indentation curve]

**Figure A.3.** An example of a force-indentation curve generated with AFM.

1. Place a clean bare glass microscope slide on the sample holder.
   a. Draw a hydrophobic circle on the glass slide before loading.
2. **Mount the probe** on the probe holder.
   a. The probe should not be too far back or too far out.
   b. It should be about 1-1.5 mm away from the end of the clear probe mount.
   c. From the side, the probe sits at a slightly slanted upward angle.
   d. Once ready, place the probe holder into the AFM head.
3. **Laser Alignment.**
   a. Find the laser spot and place it at the end of the tip to be used.
   b. Adjust to get maximum sum signal.
   c. Adjust deflection to zero.
   d. Let it stand for a few minutes if deflection fluctuates. Deflection may be unstable if there are charges on the surface or huge adhesion forces near the surface due to a water layer on a rainy day, etc.
4. **Check thermal peaks** under the thermal tab. Verify that a correct peak exits within the manufacturer specified drive frequency range.
5. **Force calibration.**
   a. ‘Engage’. Zero the deflection then bring down the head until the tip touches the surface, with a clicking sound from the software. Adjust Z voltage to be at zero.
   b. Adjust settings: Trigger Channel
      i. Trigger channel: DefInVols
      ii. Positive slope
      iii. Relative
      iv. For soft cantilevers, change Force distance = 10 um
Trigger point = 3 V

c. Hit ‘Single Force’. It should generate a single force curve. Hit it one more time if the tail is too long or is at an angle.
   i. Bring out cursors with “Control + i”
   ii. Locate them on the tail
   iii. Set sensitivity = Virtual Deflection
   iv. Zoom into the linear region of the curve.
   v. Place the cursors on the linear region. The cursors should move together if you move along the curve with arrow keys.
   vi. Set sensitivity = DeftInVols
   vii. Check AmpInVols to see if it’s between 30 and 60. Write down this number. Even if this number is greater than 60, it is okay as long as the calibrated spring constant is close to the nominal spring constant.

6. **Thermal calibration**.
   a. Physically lift head up from sample (turn the knob 10x). Deflection should change as the head is moved up; however, it should stay stable.
   b. Do thermal.
   c. Fit to the peak corresponding to the specified drive frequency of the tip.
   d. ‘Try fit’ until little to no change in spring constant. Record the spring constant.
   e. Check AmpInVols. Record this number.

7. **Transition to Liquid Mode**.
   a. Carefully lift head up.
   b. Add a few drops of water on the probe and around it.
   c. Pipette some DI water onto the pre-drawn hydrophobic circle on glass slide.
   d. Place the head down carefully. You can see from the camera that the tip is now submerged in water.
   e. Make sure there are no air bubbles on the tip!
   f. Adjust deflection and laser position to get good sum signal.
   g. Wait 5 minutes or more for deflection to stabilize.
   h. Do ‘Single force’ to create a force curve. It should look similar to the force curve in air mode.
   i. Check AmpInVols. It may shift quite a bit. Record this number. You will use this number for any subsequent data analysis.

2. **Force measurements of the gel samples**

1. Make sure head is level. Also, disengage head so that the tip won’t be touching the sample once the sample is loaded. The tip will get destroyed if head is too close to sample.

2. **Load sample**.

   NOTE: Always do the stiff substrates first, then move to softer ones to minimize damage to AFM probes.

   a. Bring head down so that the tip is fully immersed in liquid.
   b. Wait about 5 minutes for deflection to stabilize.
   c. Engage the tip and find the surface, then withdraw.
   d. Adjust force measurement settings.
      i. Velocity = 1 µm/s
ii. Indentation depth = 2 µm
   
e. Hit Single force.
   
f. Hit withdraw.
   
g. Move the stage in x- and/or y-directions to locate the AFM probe on different spots of the loaded samples. Repeat e-f. (N = at least 15 per sample)

8. When done, lift up the head.
9. Remove the sample. Load another and repeat as needed.

3. Saving the data files

1. Force tab -> ‘Review’.
2. Select force curves to be saved.
3. Modify x- and y-offset.
4. Menu on the very top of IGOR Pro: Select Data -> Save waves -> Save as delimited text.
5. Find the folder where the force data is saved.
6. Select the correct files to be imported then hit ‘Do it’. For Hertz force calculations, only the ‘Ext’ files are required. (Red = extension; Blue = retraction).

4. Clean-up

1. Remove the AFM probe and clean the probe holder with an air spray or nitrogen spray to remove any liquid. Put the holder back into the head.
2. Put everything back to where it was.
3. Keep the AFM chamber closed.
APPENDIX B: CELL CULTURE AND ANALYSIS PROTOCOLS

B.1. HSC Isolation Protocol

REFERENCES: (294)

SUPPLIES

Animals to sac:
WT Jax C57B6 mouse strain: 1 mouse

FACS Antibodies:
Lineage cocktail [CD5, CD45R (B220), CD11b, Anti-Gr-1 (Ly-6G/C), 7-4, Ter-119]
Sca-1
c-Kit
*All antibodies are purchased from eBiosciences.

Materials:
70% ethanol solution
Surgical tools – scissors, tweezers
Timer; Pen; Petri dish
Vacuum chamber – vacuum flask, plastic tubing, and glass pipettes for aspiration
Pre-cooled reagents – ACK lysis buffer, separation buffer, PBS/2%FBS (at 4°C)
PBS/2%FBS: 20 mL for temporarily storing femurs and tibias
40 mL for crushing and filtering bones
35 mL for quenching after lysis step
10 mL for miscellaneous use
40 µm cell strainer

EQUIPMENT
Biological safety cabinet; Vortex; Centrifuge; Flow cytometer (Analyzer or sorter)

PROCEDURE

Isolation of BM cells from mouse:

1. Sacrifice mouse via CO₂ inhalation. (Make sure to bring 70% ethanol solution, surgical scissors and tweezers, gloves, and a pen to the animal facilities lab.)
2. Remove tibia and femur (entire length incl. head of femur) from both sides. Remove any attached muscle (via gentle Kim-wipe rubbing). Keep cleaned bones in PBS+2%FBS.
3. Place bones into (cleaned/autoclaved) mortar with some PBS+2%FBS. Gently crush the bones using a pestle. Do not grind with continuous motion in the x direction: instead, use a gentle up-and-down motion.
4. Pour PBS+2%FBS + ground bones through a 40μm cell strainer into a 50ml conical tube. Add more PBS+2%FBS and grind. Repeat until 40mL PBS+2%FBS is used up. While doing this, also make sure to rinse mortar and pestle once in a while to collect all cells.
5. Spin down: 1400 rpm, 5 minutes, 4°C. Aspirate supernatant.
6. Resuspend cells in 5ml ACK lysis buffer, vortex well. Let stand 5 min (no more!) on ice. Quench with 35ml PBS+2%FBS.
7. Spin down: 1400 rpm, 5 minutes, 4°C. Aspirate supernatant.
8. Resuspend in PBS+2%FBS in 1mL. Count live cells from a 10μL aliquot (+10μL Trypan blue) with a hemocytometer. For easy cell counting, dilute the cell suspension at least by a factor of 5.

<table>
<thead>
<tr>
<th>WT</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Avg. count (hemocytometer grid)</td>
<td></td>
</tr>
<tr>
<td>Total # cells/mL</td>
<td></td>
</tr>
</tbody>
</table>

**FACS analysis/sorting for further enrichment in LSK population: All steps done on ice**

1. Add Fc receptor blocking antibody to achieve 0.5 μg/mL concentration. Sit 10’ on ice.
2. Spin down: 1400 rpm; 5 minutes; 4°C. Aspirate the supernatant.
3. Resuspend in 1mL PBS+2% FBS.

For first time sorting or when compensation needs to be updated, divide the BM suspension in volumes of 300μL containing 250,000 cells to generate control samples listed in the following table.

<table>
<thead>
<tr>
<th>#</th>
<th>FAC S Tube</th>
<th>Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Unstained control</td>
<td>WBM</td>
</tr>
<tr>
<td>2</td>
<td>PI control</td>
<td>WBM, PI</td>
</tr>
<tr>
<td>3</td>
<td>APC control</td>
<td>WBM, Sca-1-APC</td>
</tr>
<tr>
<td>4</td>
<td>PE control</td>
<td>WBM, c-kit-PE</td>
</tr>
<tr>
<td>5</td>
<td>FITC control</td>
<td>WBM, Lin-FITC</td>
</tr>
<tr>
<td>6</td>
<td>Stained sample</td>
<td>WBM, PI, c-kit-PE, Sca-1-APC, Lin-FITC</td>
</tr>
</tbody>
</table>

4. Add antibody cocktails (Lin, Sca-1, and c-Kit); Sit 20’ on ice.

<table>
<thead>
<tr>
<th>Lin cocktail</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD5</td>
<td>1:200</td>
</tr>
<tr>
<td>B220</td>
<td>1:300</td>
</tr>
<tr>
<td>Mac-1</td>
<td>1:320</td>
</tr>
<tr>
<td>Antibody cocktail</td>
<td>Amount</td>
</tr>
<tr>
<td>-------------------</td>
<td>--------</td>
</tr>
<tr>
<td>Lin cocktail</td>
<td>7 µL</td>
</tr>
<tr>
<td>Sca-1-PE</td>
<td>7 µL</td>
</tr>
<tr>
<td>c-kit-APC</td>
<td>7 µL</td>
</tr>
<tr>
<td>TOTAL</td>
<td>21 µL</td>
</tr>
</tbody>
</table>

Note) Dilutions when antibodies are at 0.5 mg/mL.

5. Add 100 µL of PI stock solution into 10mL PBS+2%FBS to make a PI working solution. Add 20µL of the PI working solution to the cells. Mix. Quench with 2mL PBS/2% FBS right away because PI staining is instantaneous.
6. Spin down: 1400 rpm, 5 minutes, 4°C.
7. Resuspend in 1.5mL PBS+2% FBS; pipette up/down, vortex to generate single cell suspension.
8. Filter into new tubes. Use polypropylene tubes to minimize cell loss through cells attaching to the tubes. Polypropylene is superior to polystyrene.
9. Flow analysis/sorting on each population.
B.2. Fixed Cell Staining Protocol

SUPPLIES
Samples with cells
3.7% formaldehyde
0.1% Triton X100
ITsignal FX (Invitrogen, Carlsbad, CA)
DAPI (Invitrogen, Carlsbad, CA)
Alexa Fluor® 488-phalloidin (Invitrogen, Carlsbad, CA)
Prolong Gold® (Invitrogen, Carlsbad, CA)

EQUIPMENT
Multiphoton laser scanning microscope (LSM 710; Carl Zeiss AG, Thornwood, NY)

PROCEDURE

NOTE:
- All procedures performed at room temperature.
- 100 uL solution is used at each step unless otherwise stated.
- Leave the sample in PBS for about a minute per rinse.
- Use Kimwipes to remove excess liquid for the rinsing step.
- Do not use BSA with ITsignal FX as it may interfere with the effectiveness of the product.

1. Fix sample in 3.7% formaldehyde for 15 minutes.
   Wash sample 3x with PBS.
2. Incubate sample in 0.1% Triton X100 in PBS for 15 minutes.
   Wash sample 3x with PBS.
3. Add 2 drops (~100uL) of ITsignal FX and incubate for 30 minutes. IT signal enhances sample fluorescence signal while reducing background noise.
   Wash sample 1x with PBS.
4. Dilute 5uL of methanolic stock solution (Alexa Fluor 488-phalloidin) into 200uL PBS/ITSignal FX (150uL plus a drop of ITSignal FX) for each sample. Incubate sample in this solution for 30 minutes.
5. Prepare DAPI solution (1:800 dilution). When 25 minutes of incubation has passed (5 min before completion), place the DAPI solution to the sample.
6. After full 30 min of incubation, wash sample 3x with PBS.
7. To minimize photobleaching, add a drop or two of Prolong Gold antifade reagent. Cure for 24 hours at room temperature before storing at 4 °C.
B.3. ImageJ: Cell spread area calculation protocol

SUPPLIES
Images of cells to be analyzed (either F-actin images or phase contrast images)
ImageJ (Free download available from online)

EQUIPMENT
Fluorescence or light microscope with 40x or higher magnification

PROCEDURE
1. Open either a confocal image (fluorescence) or a phase contrast image. The image should have a scale bar.

2. Analyze -> Set scale. Usually comes to 6.3 pixels per unit OR 63 pixels for 10 microns, but will vary depending on the picture size and magnification used.

Image enhancement (Necessary for phase contrast images only): Steps #3-5

3. Process -> Smooth OR Sharpen, depending on which one gives clearer, more defined edges of the objects of interest.

4. Process -> Enhance contrast. Set it to 0.7%. If this doesn’t give defined edges, try 0.001%.

5. Image -> Adjust -> Brightness/Contrast.
   Image -> Adjust -> Color balance (All colors).
   Image -> Adjust -> Threshold color -> Hue settings reduces background noise quite well. Adjust these settings until more defined edges are obtained.

6. Image -> Type -> 8-bit to make it a black & white image.

7. Image -> Adjust -> Threshold. Adjust until the objects of interest stand out and the background noise level is minimized. Hit ‘Apply’.

8. Process -> Binary -> Make binary. A pop up window will appear. Do this once more. The pop up window will not appear this time.

9. Using the wand tool, click an object of interest. If it has holes to fill, then fill them by clicking ‘Cntl+F’. Fill until a uniformly filled object is selected.
10. **Analyze -> Set measurements -> Check ‘Area’ and ‘Perimeter’**.

11. **Analyze -> Analyze particles -> Adjust range (usually 10 – Infinity works. Increase/Decrease depending on expected cell size). Show ‘Outlines’**.

12. **Continue analyzing until only one object (or objects of interest) is identified. That is, a single object of interest being identified as multiple particles is NOT good. One cell should be identified as one particle. Record computed area and perimeter for further calculations.**

13. **Save the thresholded image for record.**
REFERENCES


