MICROSCALE MACHINING AND MECHANICAL CHARACTERIZATION OF BONE TISSUE

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ABSTRACT

Bone is an anisotropic, hierarchically structured material, and as a result, its mechanical behavior is highly statistical in nature. It has been shown for other engineering materials that mechanical testing at the microscale enables characterization of individual microstructural components in an effort to understand their role in the macroscopic mechanical behavior. The application of such microscale testing to bone will permit modeling of the aggregate material to predict effects of age, disease, or injury on the mechanical properties, thus enabling a better understanding of the disease state.

In the present work, dual focused ion beam (FIB) and femtosecond (FS) laser micromachining techniques are employed to produce microscale mechanical test specimens of bovine cortical bone on the order of $10 - 30 \mu m$. A FIB is advantageous for micromachining pillars as it is capable of producing small scale features by applying a $Ga^+$ ion beam that penetrates and removes the surrounding material. A FS laser uses ultrashort laser pulses to ablate the material by locally heating it to its vaporization temperature, creating a plasma that is dissipated into a flowing gas. The FS laser is advantageous for micromachining of biological materials because it may be used in ambient, non-vacuum environments, making it a flexible tool for machining the bone surface while preserving its microstructure. The short pulse duration minimizes thermal diffusion and heating of the surrounding material. Prior research suggests that FS laser
machining causes very little residual damage to the surrounding bone tissue. Processing parameters and feasible specimen geometries and dimensions are discussed.

The fabrication of such pillars allows for micromechanical compression testing of time independent behavior using a modified nanoindenter with a flat punch tip. By achieving successful fabrication of micron scale pillars, it is possible to test the constitutive mechanical properties of mineralized tissue that comprises bone. The present work analyzes the mechanical testing of 20- and 30 µm nominal diameter pillars to small and large strains. Pillars tested to small strains are selectively placed within regions of interest on the bone sample surface (i.e. osteons). Modulus, strength, and modes of deformation are compared between samples.
Dedicated to my family for all of their love and support
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# TABLE OF CONTENTS

ABSTRACT .......................................................................................................................... ii

ACKNOWLEDGEMENTS ................................................................................................. v

VITA .................................................................................................................................... vi

LIST OF FIGURES ............................................................................................................. x

LIST OF TABLES ................................................................................................................ xiv

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

CHAPTER 2: BACKGROUND AND LITERATURE REVIEW .............................................. 4
  2.1 Bone: Structural Components .................................................................................. 4
      2.1.1 Sub-Nanometer – Nanometer Scale ............................................................... 4
      2.1.2 Sub-Micrometer – Micrometer Scale ............................................................. 7
      2.1.3 Macrostructure ............................................................................................... 11
  2.2 Mechanical Properties of Bone Tissue ................................................................. 12
      2.2.1 Mechanical Properties – Dependency on Hydration ................................ 12
      2.2.2 Mechanical Properties of Cortical and Trabecular Bone ..................... 13
      2.2.3 Mechanical Properties of Osteons and Trabeculae ............................. 15
      2.2.4 Mechanical Properties of Lamellae ......................................................... 18
      2.2.5 Mechanical Properties of Collagen and Apatite .................................. 19
  2.3 Dual Focused Ion Beam as a Machining Device ................................................. 20
  2.4 Femtosecond Laser as a Machining Device .......................................................... 25
  2.5 Comparison of Dual Focused Ion Beam and Femtosecond Laser .................. 28

CHAPTER 3: HISTOLOGY ................................................................................................. 30
  3.1 Histology Methods and Procedures ................................................................. 30
      3.1.1 Sample Preparation ..................................................................................... 30
      3.1.2 Histology Preparation ................................................................................ 31
      3.1.3 Histology Slides ......................................................................................... 31
3.1.4 Imaging Histology Slides........................................................................32
3.2 Histology Results and Discussion................................................................32

CHAPTER 4: SAMPLE PREPARATION AND IMAGING.................................37
4.1 Sample Preparation for Micropillar Fabrication......................................37
   4.1.1 Polishing Methods to Reveal Microstructural Features of Bone Tissue....37
4.2 Imaging Bone Tissue Samples..................................................................38

CHAPTER 5: DUAL FOCUSED ION BEAM MICROMACHINING..................40
5.1 Dual Focused Ion Beam Methods and Procedures.................................40
   5.1.1 Sample Preparation for FIB Machining..............................................40
   5.1.2 Micropillar Fabrication.................................................................41
   5.1.3 Imaging of FIB Machined Pillars....................................................42
5.2 Dual-Focused Ion Beam.................................................................43
   5.2.1 Sample Surface Protection............................................................43
   5.2.2 Micropillar Fabrication.................................................................45

CHAPTER 6: FEMTOSECOND LASER MICROMACHINING.........................48
6.1 Femtosecond Laser Methods and Procedures.........................................48
   6.1.1 Single Pulse Experiment.................................................................49
   6.1.2 Pulse Overlap Experiment..............................................................50
   6.1.3 Micropillar Fabrication.................................................................52
6.2 Femtosecond Laser Results and Discussion..........................................53
   6.2.1 Single Pulse Experiment.................................................................53
   6.2.2 Pulse Overlap Experiment..............................................................59
   6.2.3 Micropillar Fabrication.................................................................62

CHAPTER 7: MICROMECHANICAL TESTING.............................................73
7.1 Microcompression Testing Methods and Procedures..............................73
   7.1.1 Preparation of Samples and Indentor Set-up....................................73
   7.1.2 Mechanical Testing of Bone Micropillars.......................................74
7.2 Micromechanical Testing Results and Discussion....................................75
   7.2.1 Pillar Arrays Tested to Large Strain...............................................75
   7.2.2 Individual Pillars Tested to Small Strain........................................80
7.2.3 Individual Pillars Located Near Periosteal and Endosteal Surfaces Tested to Small Strain...............................................................86

CHAPTER 8: CONCLUSIONS AND SUMMARY ........................................94
CHAPTER 9: FUTURE WORK ...................................................................97
REFERENCES ..........................................................................................99
APPENDIX ...............................................................................................105

Appendix A: Comparison of Micropillar Geometry and Mechanical Testing Data...105
## LIST OF FIGURES

Figure 1.1 Structural complexity of bone tissue revealing organization from the macroscale to the sub-nanoscale .................................................................2

Figure 2.1 Illustrative representation of the organization of collagen fibrils and mineralized apatite crystals. ........................................................................6

Figure 2.2 Illustrative depiction of the osteonal structure ........................................10

Figure 2.3 Cortical (C) and trabecular (T) bone structure ........................................12

Figure 2.4 Schematic of fiber orientation for adjacent lamellae in a single osteon: (a) transverse osteon, (b) alternating osteon, (c) longitudinal osteon .............16

Figure 2.5 Schematic of the dual-beam configuration in a focused ion beam .............21

Figure 2.6 Illustration of the imaging (a), milling (b), and deposition (c) capabilities of a focused ion beam ...........................................................................22

Figure 2.7 FIB machined 1 µm GaAs pillar for future mechanical testing ...............24

Figure 2.8 Irreversible thermal damage of bone tissue resulting from 150 µs laser pulses .................................................................................................26

Figure 2.9 Trench cut in porcine femur using 900 fs laser pulses revealing clean cut walls ........................................................................................................28

Figure 3.1 Macrostructure (a) and microstructure (b) of hydrated bone tissue sample. ........................................................................................................33

Figure 3.2 Macrostructure (a) and microstructure (b) of alcohol dehydrated bone tissue sample. ..........................................................................................34
Figure 3.3 Macrostructure (a) and microstructure (b) of alcohol dehydrated bone tissue sample followed by rehydration in phosphate buffered saline.

Figure 3.4 Macrostructure (a) and microstructure (b) of vacuum dried bone tissue sample.

Figure 3.5 Macrostructure (a) and microstructure (b) of vacuum dried bone tissue sample followed by rewetting in phosphate buffered saline.

Figure 3.6 Microstructure of vacuum dried bone tissue sample revealing a macroscale crack intersecting an osteon.

Figure 5.1 Electron beam image revealing damaged pillar surface from FIB machining.

Figure 5.2 Electron beam image revealing platinum protection coating on the surface of a pillar.

Figure 5.3 Electron beam image of a 2 μm diameter pillar (initial cut) milled at 500 pA.

Figure 6.1 Schematic of the femtosecond laser system.

Figure 6.2 Single pulse ablation spots for P = 1 mW (E_p = 0.33 μJ).

Figure 6.3 Single pulse ablation spots for P = 7 mW (E_p = 2.33 μJ).

Figure 6.4 Single pulse ablation spots for P = 15 mW (E_p = 5.00 μJ).

Figure 6.5 Semilogarithmic plot of ablated diameter squared versus pulse energy. Gaussian beam radius is calculated using the slope of the logarithmic trendline (ω_0 = 2.72 μm).

Figure 6.6 Semilogarithmic plot of ablated diameter squared versus fluence. Threshold fluence was calculated by extrapolating logarithmic trendline to zero diameter (F_th = 2.6 J/cm^2).

Figure 6.7 Calculated and experimental threshold fluence for determining laser power necessary for bovine bone ablation using ω_0 = 2.72 μm. Curves above the experimental threshold fluence represent laser powers capable of vaporizing bone.
Figure 6.8 Linear line ablations for laser fluence of 2.87 J/cm\(^2\) at varying scan speed. ...59

Figure 6.9 Linear line ablations for laser fluence of 14.34 J/cm\(^2\) at varying scan speed. .60

Figure 6.10 Microchannel width versus scan speed for laser fluencies 2.87 and 14.34 J/cm\(^2\). Solid lines represent best fit curves for predicted microchannel width using \(\xi = 0.92\). ..............................................................61

Figure 6.11 Femtosecond laser produced 90 \(\mu\)m pillar array...........................................63

Figure 6.12 Femtosecond laser produced 30 \(\mu\)m pillar without air blower (a) and with air blower (b).........................................................................................................64

Figure 6.13 30 \(\mu\)m diameter pillars produced using two different FS laser ablation sequences: (a) circular ablation followed by line ablation, (b) line ablation followed by circular ablation. Both sequences were performed with laser power 4.5 mW and scan rate of 0.67 mm/s........................................65

Figure 6.14 20 \(\mu\)m diameter pillar produced using the femtosecond laser at P=4 mW, S=0.67 mm/s. .................................................................66

Figure 6.15 10 \(\mu\)m diameter pillar produced using the femtosecond laser at P=2 mW, S=0.67 mm/s. .................................................................68

Figure 6.16 15 \(\mu\)m diameter pillar produced using the femtosecond laser at P=2 mW, S=0.67 mm/s. .................................................................68

Figure 6.17 (a) Optical microscope image showing region of interest on surface of bone tissue sample, (b) optical microscope image following FS laser machining, (c) ESEM image following FS laser machining. .................................................................69

Figure 7.1 Schematic of the microcompression test system..........................74

Figure 7.2 Engineering stress-strain curves for mechanically tested 20- and 30 \(\mu\)m pillars to (a) 10% strain, (b) 20% strain, and (c) 30% strain. ........................................76

Figure 7.3 Schematic illustrating a lower probability of finding a critical flaw in smaller samples.................................................................77

Figure 7.4 Deformed 20 \(\mu\)m pillar following mechanical testing to 20% strain. ............80
Figure 7.5 Deformed 30 µm pillar following mechanical testing to 20% strain. ...........80

Figure 7.6 Engineering stress-strain curves for 30 µm nominal diameter pillars tested to small strains..........................................................81

Figure 7.7 FS laser produced 25.0 µm pillars before (a) and after (b) mechanical testing to small strains........................................................................82

Figure 7.8 Engineering stress-strain curves for 20 µm nominal diameter pillars tested to small strains..........................................................83

Figure 7.9 FS laser produced 14.42 µm pillars before (a) and after (b) mechanical testing to small strains........................................................................84

Figure 7.10 Engineering stress-strain curves for 30 µm nominal diameter pillars near periosteal and endosteal surfaces tested to small strains. .............................86

Figure 7.11 FS laser produced 21.74 µm pillar before (a) and after (b) mechanical testing to small strains........................................................................87

Figure 7.12 Engineering stress-strain curves for 20 µm nominal diameter pillars near periosteal and endosteal surfaces tested to small strains. .............................89

Figure 7.13 FS laser produced 14.34 µm pillars before (a) and after (b) mechanical testing to small strains........................................................................90
List of Tables

Table 2.1 Composition of the constituents of bone tissue in dogs. ........................................5

Table 2.2 Comparison of elastic modulus for human and bovine cortical and trabecular bone ..........................................................14

Table 2.3 Values for modulus and strength of isolated osteons in tension, compression, bending, and torsion ..........................................................17

Table 2.4 Comparison of dual focused ion beam and femtosecond laser capabilities ......29

Table 6.1 Pulse overlap and number of pulses at varying scan speeds for the femtosecond laser system used. ........................................................................52

Table 6.2 Incubation coefficient for bovine femoral bone and other common materials. .62

Table 6.3 Measured dimensions and taper obtained from the SEM images for nominal 20 and 30 μm diameter pillars.................................................................67

Table 6.4 Measured dimensions and taper obtained from the SEM images for nominal 20 and 30 μm diameter pillars selectively placed within osteons. .........................71

Table 6.5 Measured dimensions and taper obtained from the SEM images for nominal 20 and 30 μm diameter pillars selectively placed within osteons at the periosteal and endosteal surface. .................................................................71

Table 7.1 Modulus and 0.2% offset yield strength for 20- and 30 μm pillars mechanically tested to large strains. .................................................................79

Table 7.2 Modulus and 0.2% offset yield strength for 20- and 30 μm pillars mechanically tested to small strains.................................................................85
Table 7.3 Modulus and 0.2% offset yield strength for 20- and 30 µm pillars mechanically tested to small strains. .................................................................................................................................91

Table A.1 Comparison of experimental pillar geometries............................................105

Table A.2 Comparison of experimental mechanical data and published mechanical data. ........................................................................................................................................................................106
CHAPTER 1
INTRODUCTION

Bone tissue is a living, continuously developing mineralized tissue that comprises the skeletal system of the body and is largely responsible for mechanical support [1]. Injury, disease, and increasing age compromise the mechanical integrity of bone tissue, leading to a decline in the mechanical properties which can result in significant impairment, discomfort, and financial concern [2, 3]. Osteoporosis is one such disease, rendering a decrease in bone mineral density which emanates an increased risk of fracture, primarily in the elderly. The National Institutes of Health estimates that 10 million people in the United States are already burdened by the disease, while another 34 million individuals are at risk for developing the disease [4]. The economic impact of osteoporosis and related bone breaks is conjectured to total $14 billion each year [4]. With the aging baby boom generation and a gain in life expectancy, the population of elderly individuals will increase worldwide thus leading to an escalated number of osteoporotic related fractures and health care expenses [5]. Advancements in the treatment and prevention of osteo-diseases requires a better knowledge of their impact on the mechanical properties of bone.

Prior research has primarily focused on studying the mechanical properties of bone at larger size scales (hundreds of microns to a few millimeters) while little research has focused on the microscale behavior of bone tissue [6]. The architectural complexity
of bone (shown in Figure 1.1) requires studies at multiple length scales in an effort to better understand the mechanical properties. Studies at smaller length scales enables characterization of the individual components that comprise bone, thus allowing modeling of the aggregate material in an attempt to understand the bulk mechanical behavior of bone tissue [7, 8].

Figure 1.1 Structural complexity of bone tissue revealing organization from the macroscale to the sub-nanoscale [8].

This study pursues the microscale characterization of the mechanical properties of bone tissue. A dual focused ion beam (FIB) and femtosecond (FS) laser are discussed as machining techniques to produce micron- and sub-micron scale pillars. Microcompression testing of the pillars using a modified nanoindenter is examined as a means of quantifying the mechanical properties of bone tissue on the micron scale. The
limitations and advancements of this method as a means of obtaining the mechanical properties of bone tissue are presented as well as future directions of the research.
CHAPTER 2
BACKGROUND AND LITERATURE REVIEW

2.1 Bone: Structural Components

Bone is a complex, hierarchically structured living tissue that has been studied and debated for hundreds of years. As technology continues to emerge, new ideas and concepts are constructed regarding the composition and structure of bone tissue, either refuting or accepting the perceptions that previously exist [7]. An interpretation of the elaborate architecture of bone follows, depicting the structural levels from the nanometer to the macroscale.

2.1.1 Sub-Nanometer – Nanometer Scale

The sub-nanostructure of bone is described as the molecular structure consisting of three main components: the inorganic matrix, organic matrix, and water. As an example, the volumetric composition of the constituent phases in dog bone is provided in Table 2.1 as well as a breakdown of compositions within each phase, respectively. Molecular water comprises 25 percent by volume of whole bone, in which 60 vol% of the water is bounded to the organic collagen, while the remaining 40 vol% is linked to the mineral phase or loose in the open cavities [9, 10]. The remaining 75 percent by volume of bone is apportioned between the organic (32%) and inorganic (43%) matrices [10].
Table 2.1 Composition of the constituents of bone tissue in dogs [10].

<table>
<thead>
<tr>
<th>Component</th>
<th>Site or specific molecule</th>
<th>Volume %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water, 25%</td>
<td>Bonded to collagen</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Other</td>
<td>40</td>
</tr>
<tr>
<td>Organic matrix, 32%</td>
<td>Collagen</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>Proteoglycan</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Other organic molecules: e.g.,</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>osteocalcin, &lt;1%; osteonectin, &lt;1%</td>
<td></td>
</tr>
<tr>
<td>Apatite mineral, 43%</td>
<td>In gaps between collagen ends</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>Intrafibrillar</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>Interfibrillar</td>
<td>14</td>
</tr>
</tbody>
</table>

The inorganic matrix is comprised of a mineralized apatite crystal generally termed “hydroxyapatite.” However, the composition of the apatite mineral is commonly disputed among researchers. Both past and present attempts at studying the composition of the inorganic phase reveal a hydroxyapatite crystalline structure using x-ray diffraction [11, 12]. However, Rey et al. concluded using Fourier transform infrared spectroscopy (FTIR) and nuclear magnetic resonance (NMR) that mineralized bone is absent of hydroxyl groups, but contains carbonate ions and acid phosphate groups and is thus a carbonate apatite [12]. There still exists much discourse regarding the assimilation of the carbonate ion into the apatite crystal, but it is recognized that it is not a true hydroxylapatite lattice [13].

Apatite crystals growing naturally in the Earth’s crust are seen to grow in a needle-like pattern, while carbonate apatite occurring in human bone tissue forms in a plate-like fashion [13, 14]. Wopenka and Pasteris believed that the incorporation of the carbonate ion into the apatite lattice alters the growth morphology thus generating the platelets [13]. The inorganic apatite crystals on average approximate 50 nm in length, 25 nm in width, and 2-3 nm in thickness [7, 8, 14]. Wopenka and Pasteris presume that the
nanometer size of the apatite crystals is the result of the increased convergence of carbonate ions [13].

The organic matrix of bone tissue is primarily composed of type I collagen, which serves as the “basic building block” providing an architectural and systematic frame on which bone is constructed. The collagenous structure is comprised of the tropocollagen triple helix, which is a convoluting of two $\alpha_1$ polypeptide chains and one $\alpha_2$ polypeptide chain [7, 15, 16]. An extracellular arrangement of tropocollagen triple helices forms tertiary structured collagen fibrils having 67 nm repeat units with 40 nm gaps as shown in Figure 2.1 [8, 16].

Figure 2.1 Illustrative representation of the organization of collagen fibrils and mineralized apatite crystals [8].
The location of the mineralized apatite crystals in relation to the collagen fibrils has long been studied. Robinson’s earlier studies concluded that the apatite crystals were contained within the amorphous cement substance and not within the collagenous fibers [11]. However, in a later study Sheldon and Robinson reasoned that the apatite exists both on the facade and within the collagen fibrils [17]. Studies have found that 40 nm holes between collagen fibrils are occupied by the mineralized apatite, while Katz and Li postulate that type I collagen provides \textit{in vivo} catalysis for precipitation of the mineralized apatite crystals [8, 18]. Overall, the self assembly of collagen fibrils forms collagen fiber networks on the order of hundreds of nanometers which are enclosed and impregnated with inorganic mineral crystals [8].

2.1.2 Sub-Micrometer – Micrometer Scale

The subsequent ultrastructural level of bone tissue consists of an almost parallel ordered alignment of collagen fibers forming what are identified as lamellae, approximately 3-7 µm in width [8]. Gebhardt (1906) conducted the earliest study of lamellar bone identifying a lateral arrangement of collagen fibrils within each lamella with a 90° rotation of collagen fibrils in the adjacent lamellae. The orientational arrangement described by Gebhardt had become the classical understanding of lamellar bone being described as a plywood structure, mimicking that of natural wood [19].

Building upon Gebhardt’s model, Giraud-Guille developed a more in depth understanding of the conformation of lamellar bone [19]. Giraud-Guille proposed two separate classifications of the collagen fiber patterns in neighboring lamellae. The first,
termed “orthogonal plywood,” follows Gebhardt’s understanding of lamellar structure, thus having orthogonal relations between contiguous collagen fibers. The second, denoted “twisted plywood,” consists of continuous nested arcs of collagen fibers possessing an infinite number of orientations [19]. Again, this time building on Giraud-Guille’s model, Weiner et al. [20] established a molecular orientation describing the appearance of the nested arc. Rather than a continuous number of angles composing the arc, they concluded a large majority of the angles to be 30°, with another prominent orientation of 70° [20]. Although a majority of researchers believe these to be the traditional views of lamellar bone structure, Marotti describes his study as being the exception. Marotti concluded through his research that the collagen fibers are interwoven and orientational differences are seen as the result of densely and loosely packed collagen regions [21].

In opposition to the well oriented lamellar bone structure, a woven bone structure may also exist, primarily in the early stages of bone development or during bone remodeling following fracture. Woven bone is comprised of a less dense arrangement of collagen fibers that have no preferred orientation. This structure is rich in non-collagenous components and contains an abundance of empty space to be later occupied by lamellar bone. Aggregates of both woven and lamellar bone produce what is called plexiform bone, present most commonly in bovine bone [14].

The well oriented lamellae form concentric circles around a medial haversian canal. Blood vessels and nerve cells are contained within the haversian canals. The overall haversian system is referred to as an osteon. Other distinctive features of the osteon include: lacunae occupied by osteocytes, canaliculi which are microscopic
passageways radiating from the central canal to the osteocytes, Volkmann’s canals running normal to the central canal, and cement lines comprised of organic material encircling the outer extremities of the osteons. Figure 2.2 shows an illustrated depiction of the structure of an osteon including all distinctive features [22].
Figure 2.2 Illustrative depiction of the osteonal structure [22].
Trabeculae represent another microstructural feature of bone comprised of dense collagen approximately 200 µm in width [23]. Trabeculae may be shaped in a rod or plate-like fashion and are frequently organized in an irregular, impinging network [8, 23].

2.1.3 Macrostructure

At the macrostructural level, bone tissue is classified in two categories: cortical (compact) bone and cancellous (trabecular) bone. Cortical bone represents the dense outer shell of bones having a porosity of only 10%. Trabecular bone is instituted in the interior portion of bone having a much greater porosity between 50-90% [22]. The differences in cortical and trabecular bone structure can be seen in Figure 2.3 revealing the densified outer cortical region and porous inner trabecular region [14]. The primary constituent of cortical bone is the osteon described above as having specific orientational arrangements of lamellae. The fundamental component of cancellous bone is the trabecula resulting in an open network. As a result of the observed macrostructural dissimilarities, there exists differences in the mechanical properties of cortical and trabecular bone [22].
2.2 Mechanical Properties of Bone Tissue

As described by its microstructural make-up, bone tissue is a hierarchically complex material structure, and as such, its mechanical properties are just as compounded. The mechanical properties of the aggregate depend on the underlying constituents and thus different testing techniques are needed to probe the mechanical properties at differing structural levels [8].

2.2.1 Mechanical Properties – Dependency on Hydration

The effects of hydration on the mechanical properties of bone have been investigated in numerous studies, concluding a definite change in the mechanical properties following removal of water [9, 24]. Rho and Pharr [24] studied the effects of drying on the modulus of interstitial lamellae and osteons in bovine bone measured by nanoindentation, finding a 9.7 and 15.4% increase, respectively. These values would
later be used as a correction for nanoindentation studies of dehydrated human bone tissue [24].

Nyman et al. [9] more extensively studied the water loss in bone tissue and its effect on strength, toughness, and stiffness. Three-point bend tests were completed on human femoral bone for both hydrated and dried specimens (vacuum drying at 21, 50, 70, and 110ºC). It was concluded that drying above 50ºC for 4 hours provided enough energy to not only remove water from open cavities, but also from the collagen and mineral matrix. The strength of bone tissue was found to initially increase following drying for 4 hours at 21ºC (5wt% water loss). However, the strength decreased following drying at greater temperatures. The toughness decreased rapidly at lower drying temperatures (least amount of water loss) and continued to decrease with an increase in drying. The stiffness of bone tissue followed a linear increase with increasing amounts of water loss. Nyman et al. [9] have theorized that water dissociates from collagen fibers at lower drying temperatures than those necessary to remove water from the mineral phase. As a result of collagen and water dissociation, the bone toughness decreases, while removal of water from the apatite mineral increases stiffness while decreasing toughness and strength [9].

2.2.2 Mechanical Properties of Cortical and Trabecular Bone

Early mechanical testing studied primarily the macrostructural properties of bone because of an inability to investigate sub-micron microstructural features. Reilly et al. [25] studied the macroscopic properties of hydrated human and bovine femoral cortical bone in both tension and compression using a load frame and an extensometer. The
values for longitudinal modulus they obtained are presented in Table 2.2. They concluded from their study that there was no statistical difference between modulus values in tension and compression and therefore list the total average modulus for bone tissue specimens [25]. The value listed for the longitudinal modulus of hydrated human femoral cortical bone (17.1 GPa) is perhaps the most widely cited value for cortical bone [26].

Table 2.2 Comparison of elastic modulus for human and bovine cortical and trabecular bone [25, 27].

<table>
<thead>
<tr>
<th></th>
<th>$E_{\text{cortical bone}}$ [GPa]</th>
<th>$E_{\text{trabecular bone}}$ [GPa]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Human Femur</strong></td>
<td>17.1 ± 3.15</td>
<td>13.0 ± 1.47</td>
</tr>
<tr>
<td><strong>Bovine Laminar Femur</strong></td>
<td>28.9 ± 6.31</td>
<td>10.9 ± 1.57</td>
</tr>
<tr>
<td><strong>Bovine Haversian Femur</strong></td>
<td>23.9 ± 5.57</td>
<td></td>
</tr>
<tr>
<td><strong>Bovine Haversian Tibia</strong></td>
<td>21.2 ± 4.15</td>
<td>–</td>
</tr>
</tbody>
</table>

Ashman and Rho investigated the mechanical properties of hydrated human and bovine femoral trabecular bone using an ultrasonic technique [27]. This material testing method propagates ultrasonic waves through the material, measuring the velocity to calculate elastic modulus. The values for modulus are provided in Table 2.2 for comparison to the values obtained for cortical bone. The results obtained for the modulus of trabecular bone are lower than those values for cortical bone. Ashman and Rho further conclude that the modulus they achieved using the ultrasonic technique correlate well with more traditional mechanical testing techniques such as buckling [27].
The values obtained from the previous studies represent the overall modulus of cortical and trabecular bone; however, mechanical properties have been shown to differ between bones and among the diverse locales of a bone [8]. For cortical bone, the mechanical properties were found to differ between specimens obtained from the tibia, femur, and humerus, while there was no such variance in trabecular bone. In cortical bone samples, the modulus was found to differ lengthwise with little discord around the perimeter. Trabecular bone samples also revealed a difference in modulus lengthwise and showed a difference around the periphery [8, 28].

2.2.3 Mechanical Properties of Osteons and Trabeculae

Ascenzi and his colleagues have extensively studied the mechanical properties of individual osteons obtained from human femoral cortical bone [29-32]. Specimens were obtained wet and machined with the haversian canal traversing the center of the osteon being sure to avoid disparities in the structure such as Volkmann’s canals. Testing was completed in a hydrated environment and included tension, compression, bending, and torsion tests. All studies compared the mechanical properties of “longitudinal osteons,” those in which the fibers ran longitudinally in adjacent lamellae, and “alternating osteons,” those in which the fibers in adjacent lamellae are oriented at 90° to each other [29-32]. In addition to these orientations, compression tests included mechanical data from “transverse osteons,” those in which the fibers run transversally in adjacent lamellae. A schematic of the fiber orientational arrangements within osteons is shown in Figure 2.4 [30].
Figure 2.4 Schematic of fiber orientation for adjacent lamellae in a single osteon: (a) transverse osteon, (b) alternating osteon, (c) longitudinal osteon [30].

Mechanical property data for the tension, compression, bending, and torsion tests completed by Ascenzi et al. is shown in Table 2.3 [29-32]. Results from tensile testing using an extensometer showed that both Young’s modulus and ultimate tensile strength are larger in osteons with longitudinally oriented lamellae [31]. Results from compression testing using an extensometer showed that in contradiction to tensile testing, the modulus and strength was greatest in transversally oriented osteons. Longitudinally oriented osteons made for the lowest modulus and strength in compression, while alternating osteons had medial values of modulus and strength. Compression tests further revealed the mode of fracture of osteon specimens was the result of crevices forming at 30-35° to the longitudinal pole of the osteon regardless of lamellar orientation [30].
Table 2.3 Values for modulus and strength of isolated osteons in tension, compression, bending, and torsion [29-32].

<table>
<thead>
<tr>
<th>Osteon Type</th>
<th>Modulus [GPa]</th>
<th>Strength [MPa]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Longitudinal</td>
<td>Alternating</td>
</tr>
<tr>
<td>Tension [31]</td>
<td>11.7 ± 5.8</td>
<td>5.5 ± 2.6</td>
</tr>
<tr>
<td>Compression [30]</td>
<td>6.3 ± 1.8</td>
<td>7.4 ± 1.6</td>
</tr>
<tr>
<td>Bending [29]</td>
<td>2.3 ± 1.2</td>
<td>2.7 ± 0.9</td>
</tr>
<tr>
<td>Torsion [32]</td>
<td>23.2 ± 7.7</td>
<td>17.2 ± 3.4</td>
</tr>
</tbody>
</table>

Three-point bending test results revealed that alternating osteons tended to have larger amounts of stiffness as the result of the transverse lamellae present. Bending of alternating osteons showed fast crack growth traversing the entire sample, resulting in minute amounts of deformation prior to failure. Longitudinal osteons, on the other hand, showed significant amounts of deformation prior to failure with a majority of the deformation occurring at the regions of tension in the bending sample [29]. Results from torsion testing showed that longitudinally oriented osteons tended to have a higher resistance to torsion and showed different modes of rupture than alternating osteons. A comparison of all testing techniques shows no one osteonal orientation dominate in withstanding tension, compression, bending, and torsion [32].

Mechanical testing has been completed on single trabeculae for comparison to the macroscopic properties of trabecular bone. Rho et al. studied the mechanical properties of single trabeculae isolated from human tibia using ultrasonic and microtensile testing.
Ultrasonic techniques were completed in a hydrated environment revealing a modulus of 14.8 ± 1.4 GPa. Microtensile testing was completed in a dry environment giving a modulus of 10.4 ± 3.5 GPa; however, with a correction for dehydration and glue (used in testing apparatus), the modulus is adjusted to 7.6 GPa. Rho et al. [33] concluded a disagreement between the two testing techniques may be the result of strain rate dependence for Young’s modulus. Furthermore, they report that deviations in specimens (i.e. age, position, and disease) may prevent a distinct value of modulus from being reported [33].

Rho et al. further studied the mechanical properties of single trabeculae using nanoindentation [34]. For these studies, measurements were made on both longitudinal and transverse trabeculae obtained from human thoracic and lumbar vertebrae in a dry environment. Rho and his colleagues found that the modulus was 19.4 ± 2.3 and 15.0 ± 2.5 GPa for longitudinal and transverse trabeculae, respectively. Based on a previously studied 15% increase in modulus for dried bone tissue, the longitudinal modulus could be corrected to 16 GPa. It is concluded that differences in modulus between longitudinal and transverse trabeculae reveal anisotropy in the material. Values for single trabeculae are still larger than macroscale values for trabecular bone, possibly due to an avoidance of flaws in the structure [34].

2.2.4 Mechanical Properties of Lamellae

Rho et al. investigated the mechanical properties of lamellae using the nanoindentation technique because of its ability to selectively test features down to 1 μm
in resolution [8, 34]. In their study, they measured the properties of osteonal and interstitial lamellae obtained from dehydrated cortical bone of human tibia. They considered both the longitudinal and transverse directions, but were unable to discriminate between transverse osteonal and interstitial lamellae, therefore providing only one value for modulus in the transverse direction. Young’s modulus was greatest in longitudinal interstitial lamellae (25.7 ± 1.7 GPa), medial in longitudinal osteonal lamellae (22.4 ± 1.2 GPa), and lowest in transverse osteonal/interstitial lamellae (16.6 ± 1.1 GPa). It is concluded that interstitial lamellae perhaps have greater amounts of mineral because they represent older bone tissue (not newly formed), and therefore have a greater stiffness. Also, it is noticed that again the longitudinal direction has a higher stiffness than the transverse direction. It should be noted that these are dry measurements, and therefore the in vivo properties may be lower in value [34].

2.2.5 Mechanical Properties of Collagen and Apatite

Mechanical properties of collagen fibers and apatite crystals have not been studied within bone tissue because of an inability to test on the nanometer and sub-nanometer scale. However, modulus values exist for collagen and mineral based on other mechanical tests. For instance, a stiffness value for collagen was obtained by tensile testing tendons composed primarily of collagen and found to be approximately 1 GPa [35, 36]. The modulus of apatite is reported at 137 GPa [37]. It is seen that the stiffness of apatite is much greater than that of bone (17.1 GPa), while the stiffness of collagen is much less. Apatite is concluded to provide stiffness to bone as a whole, while collagen supplies an increased toughness (larger strain to failure) [36].
2.3 Dual Focused Ion Beam as a Machining Device

Focused ion beam (FIB) technology has been widely used by researchers for a variety of purposes and on a number of different materials. FIB machines were initially acclaimed by the semiconductor industry for their ability to fabricate devices, including integrated circuits, repair photomasks, and prototype circuits [38, 39]. However, a FIB is functional in an array of material science applications including imaging and micromachining. FIBs may be used for tomographical studies (i.e. serial sectioning) to analyze the three-dimensional structure of a material [40]. They are also used in the preparation of transmission electron microscopy samples [41]. FIBs may be used for micro- and nanomachining through removal of the substrate material, producing for example, small scale pillars for relevant mechanical testing [42].

A FIB may be a single-beam or dual-beam instrument, the single-beam consisting only of an ion column for imaging, milling, and deposition, while the dual-beam contains an electron column for additional imaging. A schematic of the dual-beam set-up is illustrated in Figure 2.5. The ion column contains a liquid metal ion source, most commonly gallium. An electric field is applied to the needle causing the gallium to form a Taylor cone [43]. The resultant ion beam is enhanced through a series of lenses and apertures before it is incident on the sample surface with a resolution of 10 nm or less [43, 44]. Beam energy and current may then be defined by the user depending on the application and material to be examined [44].
Imaging may be completed using the ion beam by detection of secondary emitted ions and electrons from the surface of the sample [43, 44]. Ion beams have been shown to generate lower resolution images, but have enhanced contrast compared to conventional SEMs [43]. Material removal is completed with the ion beam by scanning the beam over a selected region of the sample with a predetermined pattern, resulting in sputtering of the material out of the substrate [43, 44]. Lastly, the FIB may be used to deposit a material onto the sample surface through an adsorption of precursor gases, for example platinum. An illustration of the imaging, milling, and deposition process is shown in Figure 2.6 [44].

Figure 2.5 Schematic of the dual-beam configuration in a focused ion beam [43].
Figure 2.6 Illustration of the imaging (a), milling (b), and deposition (c) capabilities of a focused ion beam [44].

Focused ion beams are advantageous in many aspects, including sample preparation, imaging, and machining. The capabilities of the dual-beam instrument, as previously discussed, are beneficial to the milling process by enabling sequential milling
and imaging without movement of the sample [45]. The excellent spatial resolution with enhanced contrast provides imaging with excellent detail. FIB instruments provide precision machining over a range of sizes (tens of nanometers to hundreds of microns), and also enable the deposition of material onto the substrate with a predisposed thickness and area [38].

However, not all aspects of the FIB are advantageous. The use of gallium as a liquid metal ion source results in gallium implantation into the surface of the sample, particularly in regions adjacent to those being imaged and machined. In addition, the gallium ion may cause damage and heat evolution to the surrounding surface, which may have adverse effects on the material substrate [43]. With respect to biological and soft materials, charging of the material surface becomes an issue as they are insulative rather than conductive surfaces [45]. Furthermore, many FIB machines operate in a high vacuum environment, thereby requiring drying of biological materials. The long milling times restrict the capabilities of producing larger scale features, which in our instance affects the milling of large trenches around the respective pillars [44].

The most applicable capability of the FIB to our research project is its ability to machine micro- and nano-sized pillars to study mechanical properties, as proposed by Uchic et al. [46]. This concept has since been applied to numerous materials in an attempt to study the size effects on mechanical properties and deformation [42, 46, 47]. An example of a micron size pillar is shown in Figure 2.7 [42]. However, to our knowledge, the process of machining micro- and nanopillars has not been applied to biological materials, particularly bone tissue.
Although FIBs have not been used for machining pillars in biological materials, literature exists discussing the use of a FIB to image biological materials and prepare TEM specimens [45, 48, 49]. Giannuzzi et al. used a FIB to study osseointegration in bone/dental implants through TEM samples and serial sectioning [49]. Hoshi et al. discussed the use of a FIB to produce TEM foils in human dentine [48]. Both studies have discussed the necessity of applying a platinum coating to protect the surface while minimizing charging. They also discussed the use of higher beam currents for initial milling procedures followed by lower beam currents for more precise milling [48, 49]. These previous studies provided guidelines when determining FIB processing parameters for machining micropillars in bone tissue.
2.4 Femtosecond Laser as a Machining Device

Lasers offer many exciting advantages in the field of surgical technology as a means of replacing traditional mechanical tools [50]. Lasers are capable of minimally invasive, non-contact surgical procedures with accelerated and well-defined removal/shaping of material and minimal damage to the adjacent tissue [50, 51]. Lasers are currently used in eye surgery (i.e. LASIK) and have increasing prospects in dental and inner and middle ear surgeries [52-54]. In addition, lasers have moving expectations in knee joint replacement surgeries by replacing conventional oscillating saws [55]. We are able to ablate bone tissue to create small scale mechanical test specimens by applying the ability of the lasers to precisely cut material.

Lasers can be divided into ultrashort pulse and longer pulse lasers depending on the time frame a laser pulse is incident on the material. Ultrashort pulse lasers most commonly constitute the femtosecond regime and are shown to be more efficient and less damaging in material removal than longer pulsed lasers [56]. Longer pulsed lasers have been shown to create significant thermal damage to the surrounding tissue as the result of temperature rises and the diffusion of heat into neighboring tissues [50, 57]. Neev et al. showed that temperatures increased to 70°C after 50 seconds of ablation using 1 ns pulses on dentin, compared to a maximum temperature of 26.9°C over 70 seconds of ablation using 350 fs pulses [50]. Kang et al. showed a carbonization of bone tissue for dry ablation using 150 μs pulses as shown by the blackened area following ablation in Figure 2.8, revealing irreversible thermal damage to the bone tissue [57].
Figure 2.8 Irreversible thermal damage of bone tissue resulting from 150 μs laser pulses [57].

In contrast to longer pulsed lasers, ultrashort pulse lasers (i.e. femtosecond lasers) are incident upon the material for a much shorter time and therefore do not cause thermal or structural affliction to the surrounding bone tissue [56]. The femtosecond laser works by heating the solid material to its vaporization temperature in the focal region of the laser through absorption of laser energy [58]. Once the material reaches the vaporization temperature, plasma is formed which expands hydrodynamically resulting in irreversible breakdown and ablation of material [58, 59]. However, the irreversible breakdown of material only occurs beyond a threshold value at which point the density of plasma exceeds a critical value. The threshold value is a laser fluence threshold provided as a function of pulsewidth and is dependent on the material being ablated [58]. The ultrashort pulses of the femtosecond laser result in more rapid ablation of material than thermal diffusion and as a result, the thermal conduction to the surrounding tissue is significantly less, thus decreasing the resultant damage [59].
The advantages of using an ultrashort pulse laser versus longer pulsed lasers are numerous. In addition to the decrease in thermal damage, removal of material using the femtosecond laser is much more efficient. Ultrashort pulse lasers also tend to minimize the stresses that move through the surrounding material, therefore decreasing any structural damage. In terms of dimensional advantages, an ablation spot is equivalent in size to the diameter of the laser beam and the depth of material removal is governable within a fraction of a micron. It is therefore possible to dimensionally and geometrically control the size and shape of the ablated area produced [60].

Femtosecond laser ablation has been completed by researchers on bone tissue samples in an effort to understand its ability to cut bone. Liu and Niemz [55] studied the ablation of porcine femur using 900 fs laser pulses, concluding its ability to precisely and accurately cut trenches. Figure 2.9 shows an SEM image of the trench cut in porcine femur by Liu and Niemz revealing clean cut walls with no evidence of thermal damage [55]. Girard et al. studied the ablation of cortical bone samples concluding ablation thresholds for their specific laser used, as well as the ability of tissue cultures to maintain cell function following femtosecond laser ablation [59]. We must define the laser parameters for our system, using previous studies as a basis on which to ablate bone tissue.
2.5 Comparison of Dual Focused Ion Beam and Femtosecond Laser

As mentioned, both a dual focused ion beam and femtosecond laser have their advantages and drawbacks in terms of micromachining capabilities. Table 2.4 provides a comparison of the aspects of both instruments as well as advantages and disadvantages in terms of material processing of bone tissue specimens.
Table 2.4 Comparison of dual focused ion beam and femtosecond laser capabilities.

<table>
<thead>
<tr>
<th></th>
<th>FIB</th>
<th>FS Laser</th>
</tr>
</thead>
<tbody>
<tr>
<td>Material removed by ion beam</td>
<td>Material is vaporized by laser</td>
<td></td>
</tr>
<tr>
<td>High vacuum environment → dehydrate sample</td>
<td>Completed in ambient air → no dehydration</td>
<td></td>
</tr>
<tr>
<td>Sample needs to be conductive</td>
<td>Sample does not need to be conductive</td>
<td></td>
</tr>
<tr>
<td>Processing speed: hours/pillar</td>
<td>Processing speed: minutes/pillar</td>
<td></td>
</tr>
<tr>
<td>Pillar size range &lt; 5 µm</td>
<td>Pillar size range &gt; 20 µm</td>
<td></td>
</tr>
<tr>
<td>Accurate pillar placement</td>
<td>Difficult to accurately position pillars</td>
<td></td>
</tr>
<tr>
<td>Specimen charging</td>
<td>Little residual damage to surroundings</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER 3
HISTOLOGY

3.1 Histology Methods and Procedures

Accurate mechanical testing data requires that the bone structure remains undamaged following sample preparation and micropillar fabrication procedures. Histological examinations of bone tissue were completed in an attempt to describe any changes in the underlying microstructure of bone following drying and rewetting events. It was necessary to study dry versus wet samples due to sample preparation methods for fabrication of bone micropillars. Focused ion beam machining requires dry samples which must then be rewetted for mechanical testing, while femtosecond laser ablation tolerates fabrication of hydrated samples. Changes in mechanical properties for dehydrated samples have been discussed in the previous chapter and this work will allow for analysis of changes in microstructural features.

3.1.1 Sample Preparation

Bone specimens were obtained from the cortical regions of bovine femoral bone, supplied by Dr. Elise Morgan at Boston University. A bone tissue sample was cross-sectioned using a diamond saw into five 4 mm sections to facilitate associations between samples. The bone tissue samples were separated into five groups termed: (1) natural
(wet), (2) vacuum dried, (3) alcohol dehydrated, (4) vacuum rewetted, and (5) alcohol rehydrated. Natural samples were used as the control and were maintained in a hydrated state while being stored in a freezer at -32°C. Vacuum dried samples were dried in a vacuum oven at 40°C for 24 hours. Alcohol dehydrated samples were immersed in four alcohol interchanges (50, 80, 95, and 100% ethanol) for 45 minutes to remove all water from the bone specimen. Rewetting/rehydrating required that the vacuum dried and alcohol dehydrated samples, respectively, be submerged in a phosphate buffered saline solution pH 7.4 (simulating in vivo conditions) for 24 hours.

3.1.2 Histology Preparation

Specimens were first fixed in 10% Neutral Buffered Formalin for 48 hours and then rinsed. Samples were then decalcified in a two step process. They were placed in a rapid decalcifier, “Decalcifier S,” containing HCl and Formic Acid for 6 hours. Following rapid decalcification, they were placed in a milder decalcifier, Formic-Citric, for 24 hours. After adequate decalcification, the samples were rinsed for 30 minutes in water and placed back into Formalin for storage and transport.

3.1.3 Histology Slides

The prepared samples were sent to the Department of Veterinary Biosciences at the Ohio State University where Alan Flechtner produced the histology slides. The respective samples were embedded in paraffin wax. Tissue sections approximately 10 µm thick were cut from the paraffin using a microtome, which were then placed on
glass slides. The bone tissue sections were finally stained with a hematoxylin and eosin stain.

3.1.4 Imaging Histology Slides

Stained slides were imaged at the Ohio State University Campus Microscopy and Image Facility. A Zeiss Axioskop® light microscope using bright-field and image capture was used for viewing the stained slides.

3.2 Histology Results and Discussion

Figures 3.1 – 3.5 reveal the results of the histological examination of drying and rewetting events on the structure of bone tissue. In each Figure, image (a) represents the macrostructural characteristics of the bone tissue sample, while image (b) represents the microstructural characteristics revealed by staining.

Macrostructural images of alcohol dehydrated and rehydrated samples reveal no differences from the naturally hydrated bone tissue samples. In addition, microstructural images showing osteonal structure and characteristics reveal no differences between alcohol dehydrated and rehydrated samples and the control. It is therefore evident that alcohol dehydration and rehydration did not significantly alter the bone structure at either the macro- or microscale.

Vacuum drying resulted in concentric cracking of the bone along the lamellae and rewetting seemed to widen the concentric cracks and generate radial cracks. Figure 3.6 reveals a microstructural image of the vacuum dried sample magnified around a selected area of the crack. The crack appears to have traversed through regions of the osteons, as
it is seen intersecting the haversian canal in the image. This disruption of the osteonal structure is only seen at the macroscale cracks, while other regions of the dried and rewetted bone sample show no significant alterations in the bone structure at the microscale.

Vacuum dried samples may have cracked due to a loss of liquid volume, whereas, alcohol dehydrated samples remained intact because there was no loss of liquid volume, but rather a replacement of water with alcohol. It is noted that different dehydration techniques did not cause significant changes to the bone structure at length scales relevant for FIB preparation of micropillars; however, chemical and molecular level changes have yet to be considered.

Figure 3.1 Macrostructure (a) and microstructure (b) of hydrated bone tissue sample.
Figure 3.2 Macrostructure (a) and microstructure (b) of alcohol dehydrated bone tissue sample.

Figure 3.3 Macrostructure (a) and microstructure (b) of alcohol dehydrated bone tissue sample followed by rehydration in phosphate buffered saline.
Figure 3.4 Macrostructure (a) and microstructure (b) of vacuum dried bone tissue sample.

Figure 3.5 Macrostructure (a) and microstructure (b) of vacuum dried bone tissue sample followed by rewetting in phosphate buffered saline.
Figure 3.6 Microstructure of vacuum dried bone tissue sample revealing a macroscale crack intersecting an osteon.
CHAPTER 4
SAMPLE PREPARATION AND IMAGING

4.1 Sample Preparation for Micropillar Fabrication

Bone specimens were obtained from the cortical regions of bovine femoral bone, supplied by Dr. Elise Morgan at Boston University. The specimens were sectioned into 5mm cross-sections using a diamond saw with water as a lubricant. Samples were maintained hydrated in a frozen state by storage in a -32°C freezer prior to and following sample preparation procedures.

4.1.1 Polishing Methods to Reveal Microstructural Features of Bone Tissue

The sections were wet ground using an Allied MultiPrep® parallel polisher. Polishing was completed in a number of steps, four minutes per step, rotating the sample 90° every minute, and at a platen rotational speed of 30 rpms. Initial polishing stages used a successive series of 600, 800, and 1200 grit Allied® Silicon Carbide discs applying water as a lubricant. These industrial mesh sizes correspond to micron gradings (particles sizes) of 15, 10, and 5 µm, respectively. However, these polishing steps alone were not enough to reveal microstructural features (i.e. osteons, lamellae, haversian canals, etc.) necessary for precise placement of fabricated pillars.
Additional preparation steps for the polishing of cortical bone tissue were provided by the University of Notre Dame’s Department of Aerospace and Mechanical Engineering [61]. Subsequent polishing steps required a progressive decrease in particle size, thus producing a lustrous surface exhibiting prominent features of the bone microstructure.

A successive series of 3, 1, and 0.25 µm Allied® polycrystalline diamond compounds were used following polishing with SiC discs. Allied Green Lube® was used as a diamond extender for polishing using all particle size diamond compounds and was chosen because it is water based and therefore compatible with bone tissue. Diamond compounds containing 3 µm and 1 µm diamond particles require the use of Allied Tech-Cloth® polishing cloths, while 0.25 µm diamond particles necessitate Allied Final A® polishing cloths. The final polishing step used an alumina slurry composed of Allied® De-Agglomerated 0.05 µm alumina powder and de-ionized water. It was necessary to use Allied Final A® polishing cloths when polishing with the alumina suspension. The bone tissue specimen was rinsed in water between polishing steps to remove the abrasive particles and avoid contaminating the subsequent polishing steps.

4.2 Imaging Bone Tissue Samples

Bone tissue samples containing ablation spots, microchannels, or fabricated pillars were imaged using an environmental scanning electron microscope (XL-30 ESEM) available in the CEOF. These samples were attached to an aluminum SEM pin stub mount using silver conductive paint. ESEM imaging was conducted using a gas
secondary electron detector (GSED) and wet mode, which replaces the residual gas in the chamber with $\text{H}_2\text{O}_{(g)}$, thereby allowing imaging of hydrated biological specimens.

Detection of the gaseous secondary electrons creates a representation of the surface of the sample, thus generating images of the ablation spots, microchannels, or pillars. Images of the surface of the sample enabled characterization of any damage created during the fabrication process, which may include thermal damage to the pillar itself or the surrounding material. Surface images also enabled measurements of specific features. Such features include: the diameter of ablation spots required for single pulse experiments, width of microchannels for pulse overlap experiments, and diameters of pillars prior to and following mechanical testing. Furthermore, samples containing micropillars were tilted within the ESEM providing a 3D representation of the pillars, and thus enabling measurements of pillar height and degree of taper. Overall, characterization using ESEM imaging allowed for analysis of processing parameters and provided a basis on which to alter design parameters.
5.1 Dual Focused Ion Beam Methods and Procedures

The dual focused ion beam (FIB) is used as a micromachining tool to produce precise micro-scale pillars from a selected area on the surface of the specimen using a focused beam of Ga\(^+\) ions. To our knowledge, the FIB has never been used as a fabrication tool for producing micropillars in biological material, including bovine bone. It is therefore necessary to define the processing parameters for FIB work which include the operating voltage, beam current, size of the pillar, and the possible need for a protective coating during fabrication.

5.1.1 Sample Preparation for FIB Machining

The dual focused ion beam, used for initial micromachining, was made available by the Campus Electron Optics Facility (CEOF) in the Materials Science and Engineering Department at the Ohio State University. The FIB is a high vacuum environment and thus requires dry samples. For that reason, previously polished, frozen samples must be dehydrated. Removal of water from the bone structure was accomplished by placing the
sample in a vacuum oven at 40°C for 24 hours. The FIB further requires that samples be electrically conductive and bone tissue is not a naturally conductive material. Dehydrated samples are fixed to an aluminum SEM pin stub mount with silver conductive paint to provide a conductive medium between the stub and bone sample. Samples are then gold coated for 70 seconds at 15 mA to provide a conductive surface on all faces of the sample. Gold coating was chosen over carbon coating because the carbon, evaporated from a heated carbon filament, produces a lot of heat and it was thought that the heat exposure may alter the bone structure. Dehydrated samples were stored in a vacuum tight desiccator prior to and following FIB milling to prevent moisture in the air from penetrating the bone sample.

5.1.2 Micropillar Fabrication

Once placed within the chamber of the FIB, electron and ion images must be focused to correctly determine the height of the sample within the chamber. Eucentric height must then be achieved through a process of tilting the sample and adjusting the height in order to obtain the same view of the specimen regardless of the tilt angle of the sample. In order to ion mill, the sample must be oriented at 52° within the machine so that the surface to be machined is perpendicular to the column from which the Ga⁺ ions are emitted. Once positioned correctly, a platinum layer must be deposited on the surface of the specimen where milling will occur in an attempt to prevent damage from the impinging ion beam [48, 49]. The Pt layer must be at least the diameter of the pillar and approximately 1.5 μm thick.
After Pt deposition, the trench that surrounds the pillar begins milling using a specific pre-determined pattern consisting of concentric circles. The concentric circles all have an inner radius corresponding to the final radius of the pillar. The outermost circle must have an outer radius approximately 80 µm in diameter to allow for the microcompression tip to compress the pillar without contacting the surrounding bone tissue. The initial cut in the milling process produces a cone shaped pillar as the result of the “single-pass scanning” and redeposition [43].

After the initial (rough) cut, a second and final cut using “multi-pass scanning” mills away the sides of the cone to produce a cylindrical pillar [43]. For final milling, a fiducial mark must be placed on the top surface of the pillar for continuous recognition throughout the milling process. The fiducial mark must be half the diameter of the final pillar size and milled at a low beam current in an effort to achieve sharp lines. Line scans are then repeated around the diameter of the pillar to remove the taper.

5.1.3 Imaging of FIB Machined Pillars

An advantage of using the dual beam FIB lies in the electron beam oriented 52° to the ion beam which is used to gather SEM images at any time during the milling process. The SEM images help to determine the progress of the pillar during milling and enable images to be taken concurrently to illustrate the milling process. The SEM images along with ion beam images also assist in the correct placement of the pattern to be milled as they reveal surface features of the bone specimen, allowing for avoidance of surface cracks and placement within osteons.
5.2 Dual-Focused Ion Beam Results and Discussion

FIB machining has the advantages of producing precise small scale features and has been successfully used in producing microscale pillars in many metals and other materials. To our knowledge, the FIB has not been used as a micropillar fabrication tool for bone tissue samples, and the procedure must therefore be defined and optimized for FIB machining of bovine femoral bone.

5.2.1 Sample Surface Protection

Fabrication was first carried out on an unprotected area of the bone surface (only gold coated), resulting in the damaged surface shown in Figure 5.1. The resultant surface has been damaged in a pitted manner creating a porous looking structure as the result of ion damage due to the penetration of Ga\(^+\) ions. Bone tissue is a naturally porous material; however, the surface should include characteristic features of the osteonal structure rather than the pitted type of damage shown. As the Ga\(^+\) ion beam continually rasters over the unprotected bone tissue surface, the surface will gather increasing amounts of damage, rendering the pillar useless for future machining or mechanical testing. It is therefore necessary to protect the surface from ion beam penetration in an attempt to minimize damage.
In an effort to prevent damage to the bone tissue surface, a platinum protective layer was applied in a circular pattern having a diameter at least equal to the diameter of the pillar. FIB milling was then completed around the platinum layer, leaving only the platinum coating on the surface of the resulting pillar. Figure 5.2 reveals the presence of the platinum protective coating on the top surface of the micropillar following initial cutting procedures.
It is apparent that the platinum layer successfully protects the bone as it is seen that the surface is flat and smooth in comparison to the porous damaged structure shown in Figure 5.1. A future issue centering on the presence of the platinum layer will involve its effect on the mechanical properties obtained by compressing the bone micropillars. Platinum has a Young’s modulus of 168 GPa, much greater than cortical bone, and a tensile strength of 125 - 240 MPa, similar to cortical bone [62]. The mechanical testing data could be corrected in knowing the values for platinum, thereby providing data for the cortical bovine bone.

5.2.2 Micropillar Fabrication

FIB machining was completed by altering the beam current (500 – 20,000 pA) and pillar diameter in an effort to achieve the best-suited micropillar for final milling procedures. The resulting micropillar following initial cut procedures should be tapered
and possess a flat parallel top surface. It was found that initial cutting procedures were successful in producing 2 μm diameter pillars at a beam current of 500 pA. The resulting micropillar is shown in Figure 5.3 and is characterized by smooth tapered sides and a flat top surface protected by the platinum layer.

![Figure 5.3](image)

Figure 5.3 Electron beam image of a 2 μm diameter pillar (initial cut) milled at 500 pA.

However, it was found that an 80 μm diameter trench would need to be milled around the pillar to accompany the microcompression flat punch tip. It was therefore necessary to use a beam current of 20,000 pA for the initial cut in an effort to cut down on milling time. A beam current of 20,000 pA produces damage on the sides of the tapered pillars, but it was thought that the final cut procedure, completed at a lower beam current (~500 pA), would remove such damage.

Attempts were made using the final cut procedures to remove the taper on the previously mentioned pillars, but these were unsuccessful. Fiducial marks were applied
to the top surface of the pillar, but were blunted and removed due to ion beam impingement on the top surface following a single pass. Without recognition of the fiducial mark, no further milling procedures could be completed. As a result, further work must be completed to better define the parameters for the final cut procedure.
6.1 Femtosecond Laser Methods and Procedures

Femtosecond (FS) laser micromachining was completed at Nanotech West Laboratories at the Ohio State University with the assistance of Dr. Dave Farson and a graduate student, Yong Chae Lim. Machining was performed using a Ti-sapphire chirped pulsed amplified FS laser (Clark-MXR-CPA-2160). A schematic of the laser is shown in Figure 6.1. The laser had a wavelength of 775 nm and a raw beam diameter of 5 mm, with a focused beam diameter of 5.44 µm. Optics were used to adjust the power of the laser beam to a desired value and a 0.42 numerical aperture optical microscope objective lens (M plan Apo NIR 50x, Mitutoyo) was used to focus the laser and identify an area of interest on the sample to be machined. Polished and hydrated samples were attached to a glass slide with double sided tape for FS laser micromachining. A computer controlled x-y-z stage (MX80L, Parker) directed the motion of the specimen under the laser, producing the desired in-plane pattern with 0.5 µm resolution.
6.1.1 Single Pulse Experiment

Single pulse experiments were performed in an attempt to analyze ablation spot size and determine the threshold laser power required for effective ablation of bovine bone. These experiments required that the laser be pulsed over the bone tissue surface at varying laser powers (pulse energies) from 1-15 mW (0.33-5.00 µJ) while creating measurable spot diameters. The equation

\[ F_{th} = F_0 \exp \left( -\frac{D^2}{2\omega_0^2} \right) \]  

relates ablation threshold fluence \( F_{th} \) to the peak fluence \( F_0 \), where \( D \) represents the diameter of the ablation spot and \( \omega_0 \) is the Gaussian beam radius [63]. In knowing the pulse energy \( E_p \) and Gaussian beam radius, the peak fluence can be calculated according to the equation.
\[ F_0 = \frac{2E_p}{\pi \omega_0^2} \]  

(2)

[63]. In a similar manner, the ablation threshold fluence can be calculated according to the equation

\[ F_{th} = \frac{2E_{th}}{\pi \omega_0^2} \]  

(3)

[63].

In executing these calculations, diameters (D) of experimental spot sizes were first imaged and measured using ESEM images and semilogarithmic plots of \( D^2 \) versus pulse energy and fluence were created. The ablation threshold fluence for bone tissue was determined by extrapolating the linear trend line to zero diameter where the ablation threshold fluence equals the peak fluence. Curves of threshold fluence for varying laser powers and spot sizes are then compared to the calculated threshold fluence to determine laser powers capable of removing bone material.

6.1.2 Pulse Overlap Experiment

Pulse overlap experiments were performed in an attempt to measure the incubation effect of multiple pulse energies impinging on the same surface area of bone tissue. Pulse overlap occurs where the number of pulses (N) is greater than 1. It has been found that incubation decreases the ablation threshold as the result of accumulation of damage from the individual pulses themselves [63, 64]. The effect of incubation must be determined for bovine bone tissue and this effect is given by the equation
where \( \xi \) represents the incubation coefficient [63].

Pulse overlap \( (O_d) \) is given by the equation

\[
O_d = 1 - \frac{s}{df}
\]

where \( s \) is the scan speed, \( d \) is the focus spot diameter (=2\( \omega_0 \)), and \( f \) is the pulse repetition frequency [63]. The number of pulses is related to the pulse overlap following the equation

\[
N = \frac{1}{1-O_d}
\]

[63].

For the laser used, \( f=3 \text{ kHz} \) and the pulse overlap and number of pulses can be calculated for a range of scan speeds and laser fluencies as shown in Table 6.1. Experimental linear line ablations were completed at laser fluencies of 2.87 and 14.34 J/cm\(^2\) and scan speeds ranging from 0.02 to 1.83 mm/s, creating measurable microchannels. The microchannel width \( (D_N) \) was measured from ESEM images and plotted versus scan speed. The incubation effect was calculated using the equation

\[
D_N = \sqrt{2\omega_0^2 \ln \left( \frac{F}{F_{th} \left( \frac{df}{\pi} \right)^{-1}} \right)}
\]

[63].
Table 6.1 Pulse overlap and number of pulses at varying scan speeds for the femtosecond laser system used.

<table>
<thead>
<tr>
<th>Scan Speed [mm/s]</th>
<th>Pulse Overlap [%]</th>
<th>Number of Pulses</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02</td>
<td>99.9</td>
<td>783.4</td>
</tr>
<tr>
<td>0.42</td>
<td>97.4</td>
<td>39.2</td>
</tr>
<tr>
<td>0.92</td>
<td>94.4</td>
<td>17.8</td>
</tr>
<tr>
<td>1.33</td>
<td>91.8</td>
<td>12.2</td>
</tr>
<tr>
<td>1.83</td>
<td>88.8</td>
<td>8.9</td>
</tr>
</tbody>
</table>

6.1.3 Micropillar Fabrication

Based on the results of single pulse and pulse overlap experiments, laser processing parameters could be optimized for micropillar production in bovine bone tissue. The fabrication of micropillars using the FS laser required the completion of preparatory codes (G codes), which involved the input of coordinates and computer codes to direct the motion of the computer controlled x-y-z stage. The in-plane shape of the pillars was controlled by the x and y coordinate components, while the z coordinate determined the height of the pillars based on the number of laser passes.

Initially 3×3 arrays of micropillars with nominal diameters ranging from 10 – 30 µm were prepared using laser powers of 2 – 4.5 mW. An air blower was used to remove debris from the area during machining. Pillar micromachining was performed in two stages. First, x- and y- line ablations were performed to produce an array of pillars with square cross-sections. The square pillars were then rounded with circular laser paths in the 2\textsuperscript{nd} stage to produce cylindrical pillars. This line-to-circular sequence was found to produce pillars with less taper than a circular only or circular-to-line sequence. Later, individual micropillars were machined in a similar manner, but were precisely placed within osteons on the bone surface.
6.2 Femtosecond Laser Results and Discussion

The femtosecond laser uses ultrashort laser pulses to ablate material by locally heating it to its vaporization temperature, creating a plasma that is dissipated into a flowing gas [58, 59]. The femtosecond laser is advantageous for micromachining of biological materials because it may be used in ambient, non-vacuum environments, making it a flexible tool for machining the bone surface while preserving its microstructure. To our knowledge, the femtosecond laser has not been used as a micromachining tool for producing micropillars in bone tissue samples. It is therefore necessary to tailor the femtosecond laser machining parameters for bovine femoral bone.

6.2.1 Single Pulse Experiment

Single pulse experiments were completed on a polished and hydrated cortical bovine bone tissue sample to determine the laser power necessary for bone ablation. These experiments involved removal of material by a single laser pulse, thus creating an ablation spot of measured diameter. Single pulse ablation spots were analyzed at varying laser powers from 1 – 15 mW, corresponding to pulse energies of 0.33 – 5.00 µJ. Figures 6.2 – 6.4 show representative images of the ablation spots for laser powers of 1 mW ($E_p = 0.33$ µJ), 7 mW ($E_p = 2.33$ µJ), and 15 mW ($E_p = 5.00$ µJ), respectively.
Figure 6.2 Single pulse ablation spots for $P = 1$ mW ($E_p = 0.33$ $\mu$J).

Figure 6.3 Single pulse ablation spots for $P = 7$ mW ($E_p = 2.33$ $\mu$J).
Diameters of the single pulse ablation spots were measured and averaged for each of the laser powers. The average ablated diameter was squared and plotted versus the pulse energy as shown in Figure 6.5 with error bars representing the maximum and minimum error from the average. The slope of the logarithmic trendline was used to calculate the Gaussian beam radius according to the equation

\[ \omega_0 = \sqrt{\frac{\text{slope}}{2}} \]  \hspace{1cm} (8)

The effective Gaussian beam radius was found to be 2.72 µm.
Knowing the Gaussian beam radius, the laser fluence was then calculated for each laser power (pulse energy) according to equation 2. The average ablated diameter squared was then plotted versus laser fluence as shown in Figure 6.6 with error bars representing the maximum and minimum error from the average. Using the equation

\[ D^2 = 2\omega_0^2[\ln(F_0) - \ln(F_{\text{th}})] \]  

and extrapolating the logarithmic trendline to zero diameter, we find the single pulse ablation threshold to be equal to 2.6 J/cm².
Using equation 1, we can plot the calculated threshold fluence ($F_{th}$) versus radius for varying laser powers, shown in Figure 6.7, in an effort to show the laser power necessary for bone ablation. The horizontal line in Figure 6.7 represents the experimentally determined threshold fluence ($F_{th} = 2.6 \text{ J/cm}^2$). The portions of the calculated curves above the experimental threshold fluence represent laser powers at which ablation should occur.
Figure 6.7 Calculated and experimental threshold fluence for determining laser power necessary for bovine bone ablation using $\omega_0 = 2.72 \mu m$. Curves above the experimental threshold fluence represent laser powers capable of vaporizing bone.

As shown in Figure 6.7, a comparison of spot diameters for a laser power of 5 mW shows excellent agreement between calculated and experimental results. The calculated fluence exceeds the experimental threshold over a 5.10 $\mu$m diameter region, while a 4.93 $\mu$m diameter hole is observed experimentally. It should be noted that, although this study indicates that bovine bone ablation will occur for laser powers greater than 1 mW, the quality of the resulting surfaces produced at this low power was poor. We observed that a laser power of 4 – 4.5 mW produced more desirable results, including smooth surface features and a depth of approximately 10 $\mu$m per pass for pillars ranging in size from 20 – 30 $\mu$m in diameter.
6.2.2 Pulse Overlap Experiment

Pulse overlap experiments were completed on a polished and hydrated cortical bovine bone tissue sample to determine the incubation effect of the femtosecond laser. The incubation coefficient, $\xi$, describes the increasing absorption of the laser pulse as the result of an accumulation of damage ($\xi = 1$ for no incubation) [63]. In knowing the value of the incubation coefficient, we can predict the width of the ablated microchannel according to equation 7, and the fluence threshold according to equation 4.

These experiments involved linear line ablations across the bone sample surface at varying laser powers and scan speeds, thereby creating microchannels of measurable widths. Microchannel widths were analyzed for laser powers of 1 mW ($F_0 = 2.87 \text{ J/cm}^2$) and 5 mW ($F_0 = 14.34 \text{ J/cm}^2$), and varying scan speeds ranging from 0.02 to 1.83 mm/s. Figures 6.8 and 6.9 show ESEM images of the microchannels created with those scan speeds and laser fluencies.

Figure 6.8 Linear line ablations for laser fluence of 2.87 J/cm² at varying scan speed.
Figure 6.9 Linear line ablations for laser fluence of 14.34 J/cm² at varying scan speed.

A plot of microchannel width versus scan speed for the two laser fluencies is shown in Figure 6.10 with error bars representing the maximum and minimum error from the average. It is seen that the channel width increases with increasing laser fluence and decreasing scan speed (increasing number of pulses). When comparing similar laser powers, the widths of the microchannels are larger than the diameters of the single pulse spots as expected due to an increasing number of pulses incident on the sample surface.
Figure 6.10 Microchannel width versus scan speed for laser fluencies 2.87 and 14.34 J/cm$^2$. Solid lines represent best fit curves for predicted microchannel width using $\xi = 0.92$.

The incubation coefficient was back calculated from equation 7 using the measured microchannel widths and found to be $\xi = 0.92$. Equation 7 was then used to predict microchannel widths for $\xi = 0.92$ and was plotted as a best fit curve shown as the solid line in Figure 4.18. For our micropillar experiments, a scan speed of 0.67 mm/s was chosen, corresponding to 24.48 pulses and a pulse overlap of 95.9%. Using equation 4, it was found that the fluence threshold for $N = 24.48$ and $\xi = 0.92$ was 2.01 J/cm$^2$ ($F_{th}^{24.48} = 2.01$ J/cm$^2$). This fluence threshold was significantly lower than the fluence threshold for a single pulse ($F_{th}^{1} = 2.6$ J/cm$^2$) due to the accumulation of damage from the increasing number of pulses.

Table 6.2 provides a comparison of the experimentally determined incubation coefficient for bovine femoral bone to some common materials, including metals and polymers [64, 65]. It is seen that in comparison to poly(methyl methacrylate) and polycarbonate, the incubation coefficient for bovine bone is much higher, while it is
slightly higher than common metals. Femtosecond laser irradiation has less of a damage effect on bone tissue than the polymers and metals listed, resulting in less permanent deformation to the material and better retaining of the bone’s natural structure.

Table 6.2 Incubation coefficient for bovine femoral bone and other common materials.

<table>
<thead>
<tr>
<th>Material</th>
<th>Incubation Coefficient, $\xi$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine femoral bone</td>
<td>0.92</td>
</tr>
<tr>
<td>Poly(methyl methacrylate) [65]</td>
<td>0.61</td>
</tr>
<tr>
<td>Polycarbonate [65]</td>
<td>0.68</td>
</tr>
<tr>
<td>Stainless Steel, 316L [64]</td>
<td>0.86</td>
</tr>
<tr>
<td>Copper [64]</td>
<td>0.87</td>
</tr>
<tr>
<td>Niobium [64]</td>
<td>0.88</td>
</tr>
<tr>
<td>Titanium [64]</td>
<td>0.83</td>
</tr>
</tbody>
</table>

6.2.3 Micropillar Fabrication

Femtosecond laser micromachining was first completed on a 3×3 array of 90 μm pillars as shown in Figure 6.11. The 90 μm pillars are cylindrical with little taper but have an aspect ratio less than the desired 3:1 (height: diameter). It is thus necessary to complete further ablation passes to produce the desired height (depth ~10 μm per pass).
Laser ablation for these pillars was completed both with and without an air blower to determine the necessity of the air blowing mechanism. A comparison of the pillars following machining is shown in Figure 6.12 (a) (without the air blower) and (b) (with the air blower). The images reveal the presence of additional debris surrounding pillars completed without the use of the air blower. It is thus essential to complete laser ablation using an air blower to produce cylindrical pillars.
The production of pillars involves two basic components employed in the G Code. The first involves circular passes that are necessary to produce cylindrical pillars, and the second includes line ablations that are necessary to remove material between the pillars. Experiments have been completed to determine the most useful combination of such G Code components to produce cylindrical 30 μm pillars. The results of these
experiments are shown in Figure 6.13 (a) (circular → line) and (b) (line → circular), both completed at a power of 4.5 mW. From these images, it is evident that to obtain the most effective pillars, line ablations must be completed prior to running the circular pattern. This process minimizes the amount of material to be removed by the circular pattern and thus decreases the chances of a resultant taper from the Gaussian beam distribution.

Figure 6.13 30 μm diameter pillars produced using two different FS laser ablation sequences: (a) circular ablation followed by line ablation, (b) line ablation followed by circular ablation. Both sequences were performed with laser power 4.5 mW and scan rate of 0.67 mm/s.
After concluding the most effective method for producing micropillars, the process was then applied to examine the capacity of the laser in producing desired dimensions, geometries, and the absence of residual damage. The femtosecond laser machined pillars with nominal diameters of 20- and 30 μm are shown in Figures 6.14 and 6.13 (b), respectively. Note that both sets of pillars have flat (non-rounded) top surfaces which are critical for maintaining direct, even contact with the flat punch tip during microcompression testing.

![20μm pillars](image)

**Figure 6.14** 20 μm diameter pillar produced using the femtosecond laser at P=4 mW, S=0.67 mm/s.

The measured dimensions and tapers for these pillars are presented in Table 6.3 as an average and standard deviation. In contrast to the 30 μm diameter pillars, the 20 μm diameter pillars have a larger taper. Both pillars have measured diameters less than the nominal diameter due to the Gaussian distribution of the laser beam which encroaches on the pillar with an increasing number (depth) of passes.
Table 6.3 Measured dimensions and taper obtained from the SEM images for nominal 20 and 30 µm diameter pillars.

<table>
<thead>
<tr>
<th></th>
<th>20µm Pillar</th>
<th>30µm Pillar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter (top of pillar)</td>
<td>17.13 (± 0.30)</td>
<td>21.81 (± 1.20)</td>
</tr>
<tr>
<td>Height [µm]</td>
<td>84.61 (± 4.01)</td>
<td>106.30 (± 2.39)</td>
</tr>
<tr>
<td>Aspect Ratio [Height:Diameter]</td>
<td>4.94 (± 0.23)</td>
<td>4.89 (± 0.31)</td>
</tr>
<tr>
<td>Taper Angle (relative to the axis of the pillar)</td>
<td>18.66° (± 1.73)</td>
<td>7.24° (± 1.04)</td>
</tr>
</tbody>
</table>

Smaller pillars with nominal diameters of 10- and 15 µm were also attempted. The smaller feature size necessitated reducing the laser power to 2 mW in an attempt to minimize damage. Representative pillars are shown in Figures 6.15 and 6.16, respectively. Both images reveal highly tapered pillars with damaged surfaces (rough surface features). Furthermore, the 10 µm diameter pillar does not have a flat top surface for the microcompression flat punch tip to contact. Due to the poor quality, 10- and 15 µm pillars were not evaluated further.
After final polishing to reveal surface microstructural features (i.e. osteons, haversian canals, etc.), attempts were made at selectively placing 20- and 30 µm pillars within osteons to achieve more accurate mechanical data of the constitutive phases of
bone tissue. The 50x optical microscope objective lens coupled to the femtosecond laser was used to identify the regions of interest on the bone sample surface. Figure 6.17 (a – c) shows the series of images prior to and following femtosecond laser machining for a 30 μm pillar. Images (a) and (b) were taken using the optical microscope while image (c) was taken using the ESEM. There is good correlation between surface features for all three images, concluding we have good control over pillar placement using the femtosecond laser.

Figure 6.17 (a) Optical microscope image showing region of interest on surface of bone tissue sample, (b) optical microscope image following FS laser machining, (c) ESEM image following FS laser machining.
The measured dimensions and tapers for the selectively located pillars are presented in Table 6.4 as an average and standard deviation. It was surprising to note that the taper angle of the 20 µm pillars was slightly smaller than the 30 µm pillars, contradicting the theory that taper angle increases as pillar diameter decreases.
Table 6.4 Measured dimensions and taper obtained from the SEM images for nominal 20 and 30 μm diameter pillars selectively placed within osteons.

<table>
<thead>
<tr>
<th></th>
<th>20μm Pillar</th>
<th>30μm Pillar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter (top of pillar) [μm]</td>
<td>14.23 (± 0.33)</td>
<td>24.26 (± 0.87)</td>
</tr>
<tr>
<td>Height [μm]</td>
<td>61.28 (± 3.92)</td>
<td>91.40 (± 3.72)</td>
</tr>
<tr>
<td>Aspect Ratio [Height:Diameter]</td>
<td>4.31 (± 0.32)</td>
<td>3.77 (± 0.10)</td>
</tr>
<tr>
<td>Taper Angle (relative to the axis of the pillar)</td>
<td>7.41° (± 0.18)</td>
<td>7.74° (± 0.13)</td>
</tr>
</tbody>
</table>

In addition to selectively placing pillars within osteons, the study was carried further, placing pillars within osteons at the periosteal (nearer the outer surface of the bone) and endosteal (nearer the marrow cavity) surface to conclude changes in mechanical properties at different regions of the bone. The measured dimensions and tapers for the selectively located pillars are presented in Table 6.5 as an average and standard deviation.

Table 6.5 Measured dimensions and taper obtained from the SEM images for nominal 20 and 30 μm diameter pillars selectively placed within osteons at the periosteal and endosteal surface.

<table>
<thead>
<tr>
<th></th>
<th>20μm Pillar</th>
<th>30μm Pillar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter (top of pillar) [μm]</td>
<td>14.24 (± 0.10)</td>
<td>23.74 (± 1.34)</td>
</tr>
<tr>
<td>Height [μm]</td>
<td>68.02 (± 2.83)</td>
<td>90.78 (± 6.15)</td>
</tr>
<tr>
<td>Aspect Ratio [Height:Diameter]</td>
<td>4.78 (± 0.18)</td>
<td>3.83 (± 0.31)</td>
</tr>
<tr>
<td>Taper Angle (relative to the axis of the pillar)</td>
<td>6.67° (± 0.72)</td>
<td>3.48° (± 0.25)</td>
</tr>
</tbody>
</table>
It has been shown for both pillar arrays and selectively placed pillars that the top diameter is less than the specified nominal diameter due to the Gaussian distribution of the beam. Selectively placed pillars (single pillars) were machined with better degrees of taper than the randomly placed arrays. Single pillars were surrounded by large trenches approximately 150 µm in diameter, whereas the pillars in an array are only separated by approximately 50 µm. The Gaussian beam is therefore only incident on the single pillar when selectively locating pillars, rather than an interaction with multiple pillars as is the case in the array. The interfacing of the laser beam with multiple pillars may be the reason for poor pillar taper in the arrays. In addition to an improvement in pillar quality when selectively locating pillars, there is also the advantage of avoiding surface features such as Volkmann’s canals and lacunae, so as to achieve more efficient pillar processing.
CHAPTER 7
MICROMECHANICAL TESTING

7.1 Microcompression Testing Methods and Procedures

Microcompression testing was completed using a Nanoindenter XP at the Air Force Research Laboratory Materials and Manufacturing Directorate (AFRL ML) located within Wright Patterson Air Force Base in Dayton, OH with the assistance of Dr. Michael Uchic and Allan Smith.

7.1.1 Preparation of Samples and Indenter Set-up

Samples were transported to the research laboratory at ambient temperature in phosphate buffered saline to maintain the hydrated state of the specimens. Compression testing was completed at room temperature (21°C) using a nanoindenter employing a goniometer stage and 50 µm diameter flat punch tip. The samples were contained within the indenter in a pre-fabricated “boat,” which consisted of an aluminum SEM pin stub mount attached to the tube portion of a single mount storage mailer filed down to the height of the bone sample. The sample was affixed within the tube of the boat and the boat was filled with phosphate buffered saline for testing within a hydrated environment (simulating \textit{in vivo} conditions). Pillars were located within the indenter using an optical microscope. Alignment of the stage and indenter tip was completed by compressing
regions of the bone sample adjacent to the pillars and altering the angle of the goniometer stage to achieve flat contact with the bone surface (a symmetric circular indent).

7.1.2 Mechanical Testing of Bone Micropillars

Mechanical testing was completed by placing the flat punch tip over the pillar of interest and applying a load, at which point, the specimen was compressed according to the defined displacement rates. A schematic of the test system is shown in Figure 7.1.

![Figure 7.1 Schematic of the microcompression test system. Image provided by Dr. Michael Uchic.](image)

Compression testing was completed on 20- and 30 µm (nominal diameter) pillars at a displacement rate of 8 and 10 nm/s, respectively. This displacement rate corresponds to strain rates of $1.33 \times 10^{-4}$ and $1.11 \times 10^{-4}$ s$^{-1}$ for the 20- and 30 µm pillars, respectively. The unload rate for mechanical testing was set at 1 µm/s. The experiments were performed in three series:

1) Pillars arranged in square arrays were tested to large strains (10-30%). Pillar arrays were located midway between the periosteum and endosteum.
2) Individual pillars were tested to small strains (testing was stopped after the first load drop on the stress-strain curve). These pillars were selectively located within osteons. The 20 µm pillars were located near the periosteal surface, while the 30 µm pillars were located near the endosteal surface.

3) Individual pillars specifically located within osteons near both the periosteal and endosteal surfaces were tested to small strains.

The ESEM was again used to image the pillars following mechanical testing in an effort to characterize the modes of deformation.

7.2 Micromechanical Testing Results and Discussion

7.2.1 Pillar Arrays Tested to Large Strain

Results for the microcompression testing of 20- and 30 µm nominal diameter pillars to 10, 20, and 30% strain are shown in Figure 7.2 (a – c), respectively. In each, the engineering stress-strain curve is compared between the differently sized pillars, with actual pillar diameters stated on the graphs. All pillars exhibited a linear, presumably elastic loading regime followed by a non-linear regime associated with damage accumulation.
Figure 7.2 Engineering stress-strain curves for mechanically tested 20- and 30 µm pillars to (a) 10% strain, (b) 20% strain, and (c) 30% strain.
The 20- and 30 µm nominal diameter pillars exhibited stress drops of approximately 10 – 15 MPa. Stress-strain curves for the 16.91- and 25.0 µm pillars tested to 10% strain are similar in shape with the smaller diameter pillar achieving higher stresses. The 23.90 µm diameter pillar tested to 20% strain exhibited a series of stress drops, suggesting a series of failure events. The engineering stress-strain curve for the 16.91 µm diameter pillar tested to 20% strain also revealed a stress drop, which was observed at the transition from linear to non-linear behavior. Data does not exist for 30 µm nominal diameter pillars to 30% strain, therefore comparisons cannot be made.

There appears to be a size effect on the failure behavior, which can be illustrated by the schematic in Figure 7.3. There tends to be a lower probability of finding a critical flaw in smaller pillars. As a result, smaller pillars may better characterize the inherent behavior of constituent phases.

Figure 7.3 Schematic illustrating a lower probability of finding a critical flaw in smaller samples.
Furthermore, there is an apparent increase in strength with deformation of the pillars, which may be attributed to the taper of the pillars. Note that the apparent increase is greater for the smaller 20 µm nominal diameter pillars, which had the higher degree of taper. A mathematical correction would need to be developed and applied to each pillar which would account for the degree of taper and provide more accurate results.

Table 7.1 provides a comparison of the average modulus and average 0.2% offset “yield” strength for both the 20- and 30 µm nominal diameter pillars randomly located and tested to large strains. Evidence of the increasing strength and stiffness of the 20 µm highly tapered pillars is seen in comparing the modulus and strength of the 20- and 30 µm pillars. However, in terms of the modulus, we cannot statistically conclude them to be significantly different at a 95% confidence level (p-value = 0.27). By contrast, in terms of the strength, there is a trend towards a statistically significant difference (p-value = 0.06). We cannot provide a direct comparison with Ascenzi and Bonucci’s study, because we have not specifically isolated osteons in this portion of the experiment. However, a simple association shows the modulus and strength for the 20 µm nominal diameter pillars to be slightly less than the compression results reported by Ascenzi and Bonucci for longitudinal and alternating osteons as seen in Table 2.3 and Table A.2, while the results for the 30 µm pillars are much less [30].
Table 7.1 Modulus and 0.2% offset yield strength for 20- and 30 μm pillars mechanically tested to large strains.

<table>
<thead>
<tr>
<th>20 μm Pillars</th>
<th>3.93 (± 0.70)</th>
<th>66.26 (± 0.30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 μm Pillars</td>
<td>5.72 (± 1.70)</td>
<td>84.12 (± 8.17)</td>
</tr>
</tbody>
</table>

Post-test ESEM images of the pillars tested to 20% strain are shown for the 16.91- and 23.90 μm pillars in Figures 7.4 and 7.5, respectively. The 16.91 μm pillar “mushroomed,” with most of the damage at the top of the pillar, where the initial cross-section was smallest. The top of the pillar flattened and billowed down on the rest of the pillar. The mushrooming effect may also contribute to the apparent increase in strength observed for the 20 μm nominal diameter pillars. The 23.90 μm pillar does not have a mushroomed top, but rather appears to have sheared off. The angle of shear is calculated to be 60.23° to the long axis of the bone, while the angle of crevices reported by Ascenzi and Bonucci was 30-35° to long axis of the bone [30]. However, it is possible that portions of the deformed pillar fell off during post-test handling, rather than during the test itself. To address this, future work would need to analyze the phosphate buffered saline solution for bone particulates.
7.2.2 Individual Pillars Tested to Small Strain

Compression testing was again completed on 20- and 30 µm pillars; however, these sets of pillars had been selectively placed within specific osteons. These tests were only carried out to small strains as they were stopped after the stress-strain response became non-linear, corresponding to the first load drop on the stress-strain curve.
Results for the microcompression testing of the 30 µm nominal diameter pillars are shown in Figure 7.6. The 30 µm pillars were located near the endosteal surface (inside surface nearer the marrow cavity), and had taper angles ranging from $7.7 - 7.9^\circ$. These pillars are seen to have similar values of stiffness as seen by the initial slope on the stress-strain curve. The 23.30- and 24.47 µm pillars reach similar stresses, whereas the 25.0 µm pillar achieves a lower stress.

![Figure 7.6 Engineering stress-strain curves for 30 µm nominal diameter pillars tested to small strains.](image)

Figures 7.7 (a) and (b) show images of the 25.0 µm pillar before (a) and after (b) testing. There are no significant shape changes following deformation of the 30 µm pillars to strains less than 5%. In contrast to the 30 µm pillars tested to large strains, these pillars did not shear or mushroom, but remained intact following deformation to small strains. Attempts to measure pillar height following deformation in an effort to compare with experimentally determined strain have yielded mixed results.
Figure 7.7 FS laser produced 25.0 μm pillar before (a) and after (b) mechanical testing to small strains.

Results for the microcompression testing of the 20 μm nominal diameter pillars are shown in Figure 7.8. In contrast to the 30 μm pillars, the 20 μm pillars were located near the periosteal surface (outer surface of the bone), and had taper angles ranging from 7.2 – 7.4°. The stiffness of the 14.42 μm pillars is similar, while the stiffness of the 13.85
µm pillar is higher. There is scatter in the stresses observed between the three pillars, and in comparison to the previous 30 µm single pillars, these pillars achieve much lower stresses.

Figure 7.8 Engineering stress-strain curves for 20 µm nominal diameter pillars tested to small strains.

Figures 7.9 (a) and (b) show images of the 14.42 µm pillar before (a) and after (b) testing. Pillar taper tends to result in localized damage at the top of the pillar. The 20 µm pillars exhibited significant shape changes after deformation resulting in material loss at the top of the pillar when tested to small strains. The strain imposed on the pillar was not enough to cause the mushrooming effect we see in pillars tested to large strains.
Figure 7.9 FS laser produced 14.42 μm pillars before (a) and after (b) mechanical testing to small strains.

Table 7.2 provides a comparison of the average modulus and average 0.2% offset “yield” strength for both the 20- and 30 μm nominal diameter pillars tested to small strains. There are differences seen in the modulus and yield strength between the 20- and 30 μm pillars, suggesting a size or location effect on the mechanical behavior. In terms of the modulus, there is a trend towards a statistically significant difference at a 95% confidence level (p-value = 0.057). In terms of the strength, we can conclude that there is
a statistically significant difference between the 20- and 30 µm pillars at a 95% confidence level (p-value = 0.049). Ascenzi and Bonucci performed compression tests on osteons from human lamellar bone for longitudinal, alternating, and transverse orientations of the lamellae, with their results reported in Table 2.3 and Table A.2 [30].

With respect to stiffness, our results for the 30 µm pillars agree more closely with these published data; however, with respect to strength, our results for the 20 µm pillars are in closer agreement. Table 2.2 suggests that bovine bone will be stiffer than human bone (macroscale results); however, our microscale results for bovine bone (30 µm pillar stiffness and 20 µm pillar strength) are surprisingly similar to human lamellar bone as seen in Table A.2.

Table 7.2 Modulus and 0.2% offset yield strength for 20- and 30 µm pillars mechanically tested to small strains.

<table>
<thead>
<tr>
<th></th>
<th>E [GPa]</th>
<th>σy [MPa]</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 µm Pillars</td>
<td>5.08 (± 0.90)</td>
<td>114.78 (± 23.11)</td>
</tr>
<tr>
<td>30 µm Pillars</td>
<td>7.48 (± 1.21)</td>
<td>202.26 (±49.09)</td>
</tr>
</tbody>
</table>

It has been shown by Rho et al. that modulus changes within the osteon, decreasing from the haversian canal to the cement line [66]. In the present work, it was difficult to obtain precise positions with respect to the haversian canal and thus differences we see in our data may be due to the differing positions within the osteons. Possible differences in strength and stiffness between the 20- and 30 µm pillars may be due to their location within the diaphysis (periosteal versus endosteal regions).
Additional experiments were next performed, controlling more accurately the placement of the pillars toward the periosteal and endosteal surfaces.

7.2.3 Individual Pillars Located Near Periosteal and Endosteal Surfaces Tested to Small Strain

Results for microcompression testing of the 30 µm nominal diameter pillars selectively placed within osteons at both the periosteal and endosteal surfaces are shown in Figure 7.10. These pillars were located near both surfaces for comparison and had taper angles ranging from 3.2 – 3.8°. The 21.74- and 24.27 µm pillars near the periosteum have similar stiffness values, but slightly different strengths. The stiffness and strength between the 24.27- and 24.66 µm pillars near the endosteum is seen to be vastly different, and is also different from those pillars near the periosteum.

Figure 7.10 Engineering stress-strain curves for 30 µm nominal diameter pillars near periosteal and endosteal surfaces tested to small strains.
Figures 7.11 (a) and (b) show images of the 21.74 µm pillar located near the periosteal surface before (a) and after (b) testing. As with the previous pillars tested to small strain, there are no significant shape changes following deformation. Again, in contrast to the 30 µm pillars tested to large strains, these pillars did not shear or mushroom, but remained intact following deformation to small strains. Attempts to measure pillar height following deformation in an effort to compare with experimentally determined strain have yielded mixed results.

Figure 7.11 FS laser produced 21.74 µm pillar before (a) and after (b) mechanical testing to small strains.
Results for microcompression testing of the 20 µm nominal diameter pillars selectively placed within osteons at both the periosteal and endosteal surfaces are shown in Figure 7.12. These pillars were located near both surfaces for comparison and had taper angles ranging from 6.2 – 7.5°. The stiffness for the 14.15- and 14.34 µm pillars near the periosteum is similar; however, they have very different strengths. The stiffness of the 14.24 µm pillar near the endosteum is higher than those near the periosteum, with an intermediate value of strength.
Figure 7.12 Engineering stress-strain curves for 20 μm nominal diameter pillars near periosteal and endosteal surfaces tested to small strains.

Figures 7.13 (a) and (b) show images of the 14.34 μm pillar located near the periosteal surface before (a) and after (b) testing. As with the previous 20 μm nominal diameter pillars tested to small strain, pillar taper tends to result in localized damage at the top of the pillar. The 20 μm pillars exhibited slight shape changes after deformation resulting in some material loss at the top of the pillar when tested to small strains. Again, the strain imposed on the pillar was not enough to cause the mushrooming effect we see in pillars tested to large strains.
Figure 7.13 FS laser produced 14.34 μm pillars before (a) and after (b) mechanical testing to small strains.

Table 7.3 provides a comparison of the average modulus and average 0.2% offset "yield" strength for both the 20- and 30 μm nominal diameter pillars located near the periosteal and endosteal surfaces tested to small strains. There are differences seen in the modulus and yield strength between the 20 μm pillars tested near the periosteal and endosteal surfaces; however, since only one pillar was tested near the endosteal surface, statistics could not be completed to determine that the difference is significant at a 95%
confidence level. There are also differences seen in the modulus and yield strength between the 30 µm pillars tested near the periosteal and endosteal surfaces; however, we cannot statistically conclude them to be significantly different at a 95% confidence level (p-value = 0.45 and 0.46 for modulus and strength, respectively). In addition, there are differences seen in the modulus and yield strength between the 20- and 30 µm pillars near the periosteal and endosteal surfaces, respectively, therefore concluding possible size effects. Statistics could not be completed for the endosteal surface as there was only one 20 µm pillar tested. The modulus is concluded to be significantly different between the 20- and 30 µm pillars near the periosteal surface at a 95% confidence level (p-value = 0.003). There is a trend towards a statistically significant difference in yield strength between the 20- and 30 µm pillars near the periosteal surface at a 95% confidence level (p-value = 0.07).

Table 7.3 Modulus and 0.2% offset yield strength for 20- and 30 µm pillars mechanically tested to small strains.

<table>
<thead>
<tr>
<th></th>
<th>E [GPa]</th>
<th>σ_y [MPa]</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 µm Pillars (Periosteum)</td>
<td>6.63 (± 0.19)</td>
<td>65.52 (± 4.58)</td>
</tr>
<tr>
<td>20 µm Pillars (Endosteum)</td>
<td>10.9*</td>
<td>94.82*</td>
</tr>
<tr>
<td>30 µm Pillars (Periosteum)</td>
<td>3.86 (± 0.12)</td>
<td>127.08 (± 24.81)</td>
</tr>
<tr>
<td>30 µm Pillars (Endosteum)</td>
<td>6.13 (± 2.76)</td>
<td>151.35 (± 28.23)</td>
</tr>
</tbody>
</table>

* Indicates only one pillar was tested and could therefore not provide an average or standard deviation.

As with the previous 20- and 30 µm pillars tested to small strain, these results can be compared to Ascenzi and Bonucci’s study on the compression testing of osteons from human lamellar bone (Table 2.3 and Table A.2) [30]. The modulus for the 20 µm pillars
located near the periosteal surface and the 30 μm pillars located near the endosteal surface are similar to Ascenzi and Bonucci’s results for the modulus of longitudinally oriented osteons. With respect to strength, our results for the 30 μm pillars (both periosteum and endosteum) and 20 μm pillars (endosteum) agree more closely with the published data. We are unable to visually identify if the lamellae in our osteons run in longitudinal or alternating orientations, and we therefore cannot accept or reject the similarity and differences seen in our results compared with Ascenzi and Bonucci’s results. Furthermore, it is not stated by Ascenzi and Bonucci as to which portion of the diaphysis their samples were obtained from, so we are unable to make cross-comparisons with respect to periosteal and endosteal surfaces.

Comparisons of the three mechanical testing experiments (pillar arrays tested to large strain, selectively located pillars tested to small strain, and pillars near periosteal and endosteal surfaces tested to small strain) shows both agreements and differences in mechanical data (Table A.2). Results from the large strain measurements are comparable to those of the small strain measurements for 20 μm single pillars and for the 20 μm pillars located near the periosteal surface, especially in terms of modulus. The 20 μm nominal diameter pillars selectively located within osteons in the second experiment were near the periosteal surface, so it is in agreement with those pillars specifically placed there. However, the array of 20 μm pillars was located midway between the periosteum and endosteum, meaning the agreement in data for the array is not due to location. 20 μm pillars selectively located and tested to small strains have strengths much lower than the 30 μm pillars, and are more comparable to the arrays of pillars tested initially. Perhaps
the pillars tested from the 20 µm array were located in areas of interest; however, we are unable to tell.

30 µm nominal diameter pillars selectively located and tested to small strains are significantly stronger than the 30 µm pillars tested from the initial array and slightly stronger than those placed near periosteal and endosteal surfaces. By selectively placing pillars within certain surface features, we are achieving more representative results of the constituent phases of bone tissue, in opposition to the randomly placed pillar arrays where we cannot quantify what phases we are testing. Although we aim for pillar locations to be within osteons, we cannot be entirely accurate when selectively placing them. If we were to have placed pillars within the interstitial lamellae, our results would have given us higher strength and stiffness values than the osteonal lamellae, as reported by Rho et al. [34]. The randomly located pillar arrays may have been located on or near cement lines, where it has been reported that mechanical properties are less, therefore concluding lower values of modulus and strength [66]. In direct comparison of the selectively located 30 µm pillars and those located near the periosteal and endosteal surfaces, periosteal and endosteal pillars had significantly lower degrees of taper, so taper angle may have attributed to the higher strength of the selectively located 30 µm single pillars.
CHAPTER 8
CONCLUSIONS AND SUMMARY

Initial histology studies have shown there to be no significant changes in the microstructure of cortical bovine bone tissue following dehydration and rehydration. FIB micromachining of dried specimens is therefore feasible as there were no significant changes in the bone structure at length scales relevant for FIB preparation.

FIB machining of micropillars has the advantages of producing small scale features, but must be optimized for machining bone tissue. It has been concluded that a platinum protective layer must be applied to the surface of the bone sample prior to FIB milling to prevent damage due to ion beam penetration. The FIB has been used successfully in the initial cutting procedures to produce tapered pillars down to 2 µm in diameter. Milling for the initial cut must be completed at a beam current of 20,000 pA to machine a large amount of material while conserving time. Attempts at final cutting procedures have been unsuccessful due to an inability to maintain the necessary fiducial mark, but it is necessary to use a lower beam current (~500 pA) to prevent damage to the sides of the pillar.

Femtosecond laser machining of bone tissue first required single pulse and pulse overlap experiments in an effort to optimize the parameters necessary for bone ablation. Single pulse experiments found the Gaussian beam radius to be 2.72 µm with a laser
threshold fluence of 2.6 J/cm\(^2\) (material is ablated at powers of 1 mW and above). Pulse overlap experiments found the incubation coefficient to be 0.92, meaning that the ablation threshold is decreased due to an accumulation of damage. However, in comparison to other common materials, bone tissue is less damaged by the femtosecond laser. For the scanning speed used for micropillar production, the threshold fluence was reduced to 2.01 J/cm\(^2\).

Femtosecond laser ablation of micropillars has the advantages of machining hydrated bone tissue specimens rapidly with minimal damage. Processing results indicate that in order to minimize taper, it is first necessary to complete line ablations to remove a majority of the material, followed by circular ablations to generate cylindrical pillars. Although the laser has many advantages in producing micropillars, there are size restrictions that prevented us from producing high quality pillars smaller than \(~20\, \mu\text{m}\) nominal diameter.

Initial mechanical testing has concluded that tapered pillars tested to large strains deformed in a mushrooming fashion with a majority of damage occurring at the top of the pillars. The 23.90 \(\mu\text{m}\) pillar exhibited a series of load drops (10 – 15 MPa), suggesting a series of failure events. Only one load drop was observed for the 16.91 \(\mu\text{m}\) pillars tested to similar strain. This size effect on the failure behavior may be associated with a lower probability of finding a critical flaw in the smaller pillars, suggesting that smaller pillars better characterize the inherent behavior of the constituent phases. Furthermore, smaller diameter pillars had an apparent increase in strength with deformation which may be attributed to the taper of the pillars.
Mechanical testing of precisely placed 30 μm tapered pillars to small strains did not deform the pillars in a mushrooming fashion, but rather they remained intact. Precisely placed 20 μm tapered pillars exhibited material loss at the top of the pillars following testing to small strains. There were differences in modulus and strength between the 20- and 30 μm pillars which may be due to their location on the bone surface (periosteum versus endosteum), or may be the result of the size effect on mechanical data.

Mechanical testing of 20- and 30 μm pillars located near periosteal and endosteal surfaces revealed differences in modulus and strength between the two locations, but statistics could not conclude the difference to be significant on a 95% confidence level. Differences in modulus and strength were also seen between 20- and 30 μm pillars, suggesting a size effect on the mechanical data.

Comparisons between all testing experiments revealed similarities between the 20 μm nominal diameter pillars tested to large strain, those tested to small strain, and those located near the periosteal surface, particularly with respect to modulus. 30 μm nominal diameter pillars selectively located and tested to small strain were significantly stronger than the 30 μm pillar arrays initially tested, and slightly stronger than the 30 μm pillars placed near periosteal and endosteal surfaces.
CHAPTER 9

FUTURE WORK

Future work pertaining to this project involves continual advancement in the machining and mechanical testing of bovine bone micropillars. We have shown the limitations that exist in using the femtosecond laser as a micromachining technique. However, the femtosecond laser may be used as a preliminary step in the fabrication of smaller mechanical test specimens. Tapered bone pillars using the femtosecond laser may be refined to cylindrical pillars using the FIB. The advantages of the femtosecond laser as a first step process is its ability to quickly machine pillars, whereas the FIB would take hours for the first cut.

Although this seems a novel method for producing well-defined micropillars, the use of the FIB to machine bone tissue still requires much work. Typical codes used to machine metals in the FIB cannot be directly applied to the machining of bone, as bone tissue is much softer and more susceptible to Ga$^+$ ion attack. As a result, we must script a new code that allows for successful machining of cylindrical pillars in bovine bone.

The differences we have seen in the modulus and strength of the 20- and 30 µm pillars will require additional mechanical testing to better understand the reason for such differences. It has been seen by other researchers that mechanical properties change within the osteon, and thus we need to be very accurate in the placement of pillars within the osteons [66].
Additional future work includes applying the machining and mechanical testing process to trabecular bone. Transmission electron microscopy foils will be used to study fracture patterns in both cortical and trabecular bone and the chemical environment of the failure zone will be studied. Once achieving micro- and nanoscale testing results, a model of the mineralized bone tissue will be constructed.
REFERENCES


Appendix A: Comparison of Micropillar Geometry and Mechanical Testing Data

Table A.1 Comparison of experimental pillar geometries.

<table>
<thead>
<tr>
<th></th>
<th>Diameter (top of pillar) [µm]</th>
<th>Height [µm]</th>
<th>Aspect Ratio (Height:Diameter)</th>
<th>Taper Angle (relative to axis of the pillar)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 µm Pillars (Array)</td>
<td>17.13 (± 0.30)</td>
<td>84.61 (± 4.01)</td>
<td>4.94 (± 0.23)</td>
<td>18.66° (± 1.73)</td>
</tr>
<tr>
<td>30 µm Pillars (Array)</td>
<td>21.81 (± 1.20)</td>
<td>106.30 (± 2.39)</td>
<td>4.89 (± 0.31)</td>
<td>7.24° (± 1.04)</td>
</tr>
<tr>
<td>20 µm Pillars (Individual Experiment 2)</td>
<td>14.23 (± 0.33)</td>
<td>61.28 (± 3.92)</td>
<td>4.31 (± 0.32)</td>
<td>7.41° (± 0.18)</td>
</tr>
<tr>
<td>30 µm Pillars (Individual Experiment 2)</td>
<td>24.26 (± 0.87)</td>
<td>91.40 (± 3.72)</td>
<td>3.77 (± 0.10)</td>
<td>7.74° (± 0.13)</td>
</tr>
<tr>
<td>20 µm Pillars (Individual Experiment 3)</td>
<td>14.24 (± 0.10)</td>
<td>68.02 (± 2.83)</td>
<td>4.78 (± 0.18)</td>
<td>6.67° (± 0.72)</td>
</tr>
<tr>
<td>30 µm Pillars (Individual Experiment 3)</td>
<td>23.74 (± 1.34)</td>
<td>90.78 (± 6.15)</td>
<td>3.83 (± 0.31)</td>
<td>3.48° (± 0.25)</td>
</tr>
</tbody>
</table>
Table A.2 Comparison of experimental mechanical data and published mechanical data.

<table>
<thead>
<tr>
<th></th>
<th>Modulus [GPa]</th>
<th>Strength [MPa]</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 µm Pillars (Array, Large Strain)</td>
<td>5.72 (± 1.70)</td>
<td>84.12 (± 8.17)</td>
</tr>
<tr>
<td>30 µm Pillars (Array, Large Strain)</td>
<td>3.93 (± 0.70)</td>
<td>66.26 (± 0.30)</td>
</tr>
<tr>
<td>20 µm Pillars (Individual, Periosteum, Small Strain)</td>
<td>5.08 (± 0.90)</td>
<td>114.78 (± 23.11)</td>
</tr>
<tr>
<td>30 µm Pillars (Individual, Endosteum, Small Strain)</td>
<td>7.48 (± 1.21)</td>
<td>202.26 (± 49.09)</td>
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<td>20 µm Pillars (Individual, Endosteum, Small Strain)</td>
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<td>3.86 (± 0.12)</td>
<td>127.08 (± 24.81)</td>
</tr>
<tr>
<td>30 µm Pillars (Individual, Endosteum, Small Strain)</td>
<td>6.13 (± 2.76)</td>
<td>151.35 (± 28.23)</td>
</tr>
<tr>
<td>Osteons, Longitudinal Lamellae (Published Data) [30]</td>
<td>6.3 (± 1.8)</td>
<td>109.8 (± 10.1)</td>
</tr>
<tr>
<td>Osteons, Alternating Lamellae (Published Data) [30]</td>
<td>7.4 (± 1.6)</td>
<td>134.0 (± 9.3)</td>
</tr>
</tbody>
</table>

*Indicates only one pillar was tested and could therefore not provide an average or standard deviation.