NON-INVASIVE CHARACTERIZATION OF BONE TISSUE AND
MODELING OF BONE AT MICRON AND SUB-MICRON LEVELS

BY

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ABSTRACT

Bone is a composite material with a hierarchical structure, spanning from a nanoscale to a whole bone level. We focus on modeling bone at two structural length scales, namely a micron level and a sub-micron level, and perform a feasibility study of using ultrasound to detect bone growth in a limb regeneration project.

At the sub-micron level, bone consists of a single lamella which is formed by a collagen fiber network with fibers aligned in a preferentially oriented direction. We model such a fibrous network computationally using finite element software Abaqus by representing fibers as Timoshenko beams. We generate a random arrangement of fibers by using a Poisson process. We investigate the effects of fiber orientation, void volume fraction and window size on the constitutive elastic response of such a beam network. We show that generally \( C_{1111} \) and \( C_{1212} \) values decrease with increasing window size, higher scattering angle, and higher void volume fraction. In addition, there is less scatter for larger window sizes, higher scattering angles and higher void volume fractions. Percolation threshold is also determined. The effect of dangling fibers on the volume fraction and the resulting constitutive response is also investigated. Three ways of estimating volume fraction of fibers are proposed. The results show that the volume fraction may be overestimated due to dangling fibers, resulting in inaccurate constitutive response.

At micron level, bone consists of osteons and interstitial lamella which are made of lamellae. We propose using classical laminate theory, namely Sun & Li’s three dimensional laminate model to model bone at micron level. The outputs are compared with nanoindentation results, obtained from literature, and other theoretical models. Overall, our modeling results are in the good agreement with experimental results reported in literature and predictions from other analytical models.

The second part of my M.S. dissertation research focuses on the non-invasive characterization of bone using ultrasound. It involves imaging frog limbs with removed large portion of long bone in a tarsus to detect and assess the quality of regenerated bone tissue. In order to determine the regions of bone, cartilage and soft tissue, a specially written program which gives the backscatter coefficient (BSC) at a certain frequency is used here. The result shows that ultrasound hold promising potential to identify bone region and therefore it can be applied to capture growth of bone tissue in-vivo. In addition to ultrasound, the feasibility of applying optical coherence tomography (OCT) to detect bone growth in frog limb is performed. Due to the insufficient depth penetration, the existence of bone cannot be confirmed using this technique.
To all the people who love me and cherish me
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CHAPTER 1
MODELING OF LAMELLA AS BEAM NETWORK

1.1 INTRODUCTION

Many biological and synthetic materials have fiber network structures. Examples include cytoskeleton of a cell [1], blood clots, biological tissues [2,3,4,5], paper [6,7], filters, textile felts [8], and insulation materials. The mechanical properties of such fibrous materials depend on many parameters including the shape, volume fraction, orientation, arrangement and mechanical properties of fibers. Spatial randomness and heterogeneity of such networks leads to scatter in their constitutive coefficients. Another important parameter is a window size used for calculations and/or testing, with considerations of that type going back to Delaunay networks [9]; the window size is typically a statistical volume element (SVE) and its scaling trend towards the representative volume element (RVE) has to be established.

All the studies of mechanical behavior of fiber networks may be classified into two main categories: (a) phenomenological models and (b) micromechanics-based models. The first type of studies is based on fitting a mathematical equation to the observed stress-strain curves of the sample. In this first category, there is oftentimes ambiguity as to how the model parameters are related to the fiber and network properties. This shortcoming is overcome by the second approach, which aims to homogenize the two-phase fiber-void material as a continuum, using either analytical micromechanics tools or computer simulations. So far, several micromechanical models have been proposed.

Focusing on the models developed to study a single lamella in bone, which is our main interest, we note the work of Hellmich [10] who developed a continuum micromechanics representation of the collagen-mineral interaction in the elasticity of mineralized tissue through a Mori-Tanaka formulation. Yoon and Cowin [11] proposed a similar model that contains cylindrically shaped collagen fibers embedded in the hydroxyapatite mineral-water composite, assuming all fibers aligned in the longitudinal direction. Both Yoon and Hellmich’s work could provide a full set of the anisotropic elastic constants of a lamella.

Computational mechanics model from the standpoint of mechanics of a random network of cellulose fibers is also proposed to represent a single lamella as a collection of mineralized fibrils in a preferentially oriented direction [12]. Other finite element models include a structural
model that uses a volume-averaging theory [13] and a representative microstructure finite element model also based on an averaging theory to model a collagen network [14]. There are also studies which treated these network-like biological tissues as semi-flexible polymers. One of them is a formulation which maps the deformation of the network to that of an equivalent continuum in order to establish a one-to-one correspondence between defects in a regular network and point sources in an equivalent continuum [15,16]. From his observation, fibers with less bending stiffness are more non-affine, i.e. the local deformation is different than the applied far-field and the degree of non-affinity decreases with increasing fiber density. Besides that, Delince and Delannay try to reconcile the results from modeling through testing on a network of metallic fibres [17]. According to their model, bounds for the components of the stiffness matrix are derived from a periodic model representing the network architecture through averaging procedure.

The objective of this study is to investigate the effects of fiber orientation, void volume fraction and window size on the constitutive response of a randomly generated fiber networks. The effect of dangling fibers on the fiber volume fraction is discussed at the end of this study.

1.2 METHODOLOGY
1.2.1 Generating meshes
A random fiber network is created in a three dimensional test box by using a Monte Carlo method. That is, for each realization of the network, we generate locations of centers of fibers and their individual orientations from a Poisson point field [12, 18]. Next, connectivity is established through nearby fibers and extended to the whole network. Several parameters will have to be chosen before the network is imported into MATLAB® [19] for preprocessing. Since in this study we are interested in the effect of window’s size, void volume fraction and fiber orientation on the constitutive response, the parameters in Table 1.1 are used.

The $l_x$, $l_y$, and $l_z$ are the lengths of box along the x, y, and z axes, respectively; $W_{\text{fiber}}$ and $T_{\text{fiber}}$ are width and thickness of the fiber, respectively; $\theta_{\text{max}}$ is the maximum scattering angle between the x-axis and fibers where the fibers will have angle between positive $\theta_{\text{max}}$ and negative $\theta_{\text{max}}$, $\text{vf}_{\text{void}}$ is void volume fraction. Given the fiber length, a dimensionless window size (or size of SVE) is defined as ratio of box length over fiber length, and denoted as $\delta$. Lastly, the variable for percolation studies is void volume fraction; the variable for orientation study is
scattering angle while the variable for window size study is fiber length. In each realization, a different seed number is chosen so that the fibers are placed differently in each run.

1.2.2 Preprocessing in MATLAB®

1.2.2.1 Input parameters

The coordinates of the nodes and the elements which constitute the network is imported into MATLAB® program. The box dimension as in Table 1.1 is entered into the program in order to determine the boundary condition later. The cross section of the fiber is chosen to be circular shape. Other input parameters are included in Table 1.2.

1.2.2.2 Trimming fibers

In the previous fiber generation process, some fibers might protrude or fall outside of the box. Therefore it is necessary to trim the fibers so that all the fibers are contained in the box. Figure 1.1 shows the regions where the fibers might fall or protrude into. The following formulations assume that the fibers do not protrude into the negative region of the boxes.

For region 1, the following equations are used:

\[
Y_{\text{new}}^2 = \frac{(l_x - X_{\text{old}}^1)(Y_{\text{old}}^2 - Y_{\text{old}}^1)}{X_{\text{old}}^2 - X_{\text{old}}^1} + Y_{\text{old}}^1 \quad (1)
\]

\[
Z_{\text{new}}^2 = \frac{(l_x - X_{\text{old}}^1)(Z_{\text{old}}^2 - Z_{\text{old}}^1)}{X_{\text{old}}^2 - X_{\text{old}}^1} + Z_{\text{old}}^1 \quad (2)
\]

\[
X_{\text{new}}^2 = l_x \quad (3)
\]

where \(X, Y, Z\) are the local coordinates of the node, superscript 1 and 2 denote node inside the test box and node outside the test box, respectively, while subscript \(\text{old}\) and \(\text{new}\) denote coordinate before and after trimming, respectively.

For region 2, the following equations are used:

\[
X_{\text{new}}^2 = \frac{(l_y - Y_{\text{old}}^1)(X_{\text{old}}^2 - X_{\text{old}}^1)}{Y_{\text{old}}^2 - Y_{\text{old}}^1} + X_{\text{old}}^1 \quad (4)
\]

\[
Z_{\text{new}}^2 = \frac{(l_y - Y_{\text{old}}^1)(Z_{\text{old}}^2 - Z_{\text{old}}^1)}{Y_{\text{old}}^2 - Y_{\text{old}}^1} + Z_{\text{old}}^1 \quad (5)
\]

\[
Y_{\text{new}}^2 = l_y \quad (6)
\]
For region 3, the following equations are used:

\[
X_{new}^2 = \frac{(l_z - Z_{old}^1)(X_{old}^2 - X_{old}^1)}{Z_{old}^2 - Z_{old}^1} + X_{old}^1
\]  
\[\text{Eqn. 7}\]

\[
y_{new}^2 = \frac{(l_z - Z_{old}^1)(Y_{old}^2 - Y_{old}^1)}{Z_{old}^2 - Z_{old}^1} + Y_{old}^1
\]  
\[\text{Eqn. 8}\]

\[
z_{new}^2 = l_z
\]  
\[\text{Eqn. 9}\]

For region 4, since we do not know a priori whether a fiber will protrude through region 1 or region 2, we determine it in the following way. We first assume that the fiber will protrude into region 4 through region 1. Equation 1 is then used to find the coordinate \( Y \) of the new node. The coordinate \( Y \) of this node is then compared with \( l_y \). If \( l_y \) is larger than new coordinate \( Y \), then the fiber is confirmed to protrude from the test box into region 4 through region 1. Therefore equations 1 through 3 are used to find the intersected node on the face of the test box. On the contrary, if \( l_y \) is smaller than new coordinate \( Y \), then equations 4 through 6 are used to find the new coordinates since the fiber will protrude into region 4 through region 2.

The same concept can be applied to the fibers protruding into region 5. Equations 4 through 6 are used if the fibers will protrude through region 2, and equations 7 through 9 are used if the fibers will protrude through region 3. Similarly for region 6, equations 1 through 3 are used for the fibers protruding through region 1, and equations 7 through 9 are used for the fibers protruding through region 3.

Region 7 is a little bit tricky since the fiber generally will go through two regions before reaching region 7. Firstly, let assume that the fiber will protrude from the test box into region 7 through region 1, and therefore equations 2 and 3 are used. If both \( l_y \) and \( l_z \) are larger than new coordinate \( Y \) and new coordinate \( Z \), respectively, then equations 1 through 3 are used since the fiber will go through region 1 and either region 4 or region 6 before reaching region 7. However, if either of \( l_y \) or \( l_z \) is smaller than the new coordinate \( Y \) or new coordinate \( Z \), respectively, then the fiber does not protrude through region 1, but through region 2 or region 3 instead. In order to determine the protruded region, let assume that the fiber protrude through region 2 and therefore equations 4 and 5 are used. If \( l_z \) is larger than new coordinate \( Z \), then the fiber protrudes through region 2 and then either region 4 or region 5 before reaching region 7. Therefore equations 4 through 6 are used. On the contrary, equations 7 through 9 are used for fiber protruding through region 3.
Note that some elements might have two nodes at the same place if the elements protrude out of test box from the faces of test box. In order to avoid this condition, the length of each element is checked so that they will have value larger than zero, which means that the node does not overlap with another.

The usage of this trimming action can be used to study the constitutive response on a certain part of the fiber network as well. Test box can be placed in a specific area of the network, while the rest of the area can be trimmed off.

1.2.2.3 Removing elements and clusters not contributing to the constitutive response.

The boundary conditions used in this study are uniaxial displacement and shear displacement (with uniform strain) at six faces of the test box. When these boundary conditions are applied on the network, some elements in the test box not touching the surface might not experience the load, resulting in overestimated volume fraction of the fibers. This will underestimate the constitutive response of the network, therefore leading to an inaccurate result. Thus, there is a need to remove any elements which do not contribute to the overall response of the network. These include an element or a cluster

1. which does not experience any load, or is known as dangling
2. at the surface where fixed displacement is applied but without any other loads
3. which only experiences a single value loading at the end surface parallel to $l_x$
4. which only experiences loading at one point

This concept has been verified through Abaqus® [20], where in each condition stated above, the strain energy for those elements is negligible compared with the overall strain energy of the fiber network system.

In order to carry out this operation, the nodes and then the elements at the six end surfaces have to be determined. Each of these end surface elements are then classified as a single cluster, and any elements that belong to this cluster are found by looping the network until no other elements are found. This process consumes most computer resources and with a decreased element length, the time it took to run the program was longer and sometimes lasted for several days. After the elements are grouped into clusters, those clusters which fulfill the conditions mentioned will be removed.
1.2.3 Issues when importing into Abaqus®

1.2.3.1 Choosing beam element

There are two types of beam that can be chosen in Abaqus®, namely the Euler-Bernoulli beam and the Timoshenko beam. The difference between these two beam elements is that there is a second order spatial derivative present in the Timoshenko beam, resulting in lower stiffness of the beam and larger deflection under loading. This characteristic prompts the selection of Timoshenko beam over Euler-Bernoulli beam in order to model fiber.

Shear coefficient for circular cross section is chosen from [21], and is defined as follows:

\[ k = \frac{6(1+\nu)^2}{7+12\nu+4\nu^2} \]  (10)

Shear rigidity, \( SR \), are then imported into Abaqus® using the following definition

\[
\begin{align*}
SR1 &= kG\pi r^2 \\
SR2 &= kG\pi r^2 \\
SR3 &= 0.25 \\
G &= \frac{E}{2(1+\nu)}
\end{align*}
\]  (11)

where \( \nu \) is the Poisson ratio, \( E \) is the elastic modulus and \( G \) is the shear modulus.

1.2.3.2 Specifying beam orientation

The orientation of a beam cross-section is defined in terms of a local, right handed system, as shown in Fig. 1.2.

In our simulation, 3D beam elements are used. An additional node off the beam axis is to be defined and included in the element’s connectivity list. Abaqus® will then be able to compute three vectors through these nodes. The dot and cross product of these vectors give the orientation of the beam [22].

1.2.3.3 Error when importing into Abaqus® due to parallel beam with z-axis.

Note that when nodes and elements are imported into Abaqus®, Abaqus® sometimes will produce the following error: THE BEAM CROSS-SECTION DIRECTION VECTORS COINCIDE AT A NODE OF ELEMENT XXX. This issue arises when the tangent vector of the beam is parallel to the z-axis of the test box. It can be solved by moving either x-coordinate or y-
coordinate of one of the node by a negligible amount compared with the distance of the node to the axis. In this case, the node is moved by a thousandth of the value of either x-coordinate or y-coordinate.

1.2.3.4 Specifying boundary conditions
In this paper we are focusing on applying uniaxial and shear displacements at the six faces of the test box in order to obtain the apparent effective coefficients $C_{1111}$ and $C_{1212}$, respectively. The nodes at the six faces of the test box are found before the loading displacement is applied. The applied uniaxial displacement to obtain $C_{1111}$ in Abaqus® is defined as follows:

$$
\begin{align*}
  u_1 &= \varepsilon_{11}x_1 \\
  u_2 &= 0 \\
  u_3 &= 0
\end{align*}
$$

(13)

where $u_i$ is the displacement, $x_i$ is the global coordinate, $\varepsilon_{ij}$ is a uniform strain, and subscript $i$ can take the value 1, 2, 3 to represent global coordinate of the fiber network. For $C_{1111}$, the uniaxial displacement is applied along 1-direction.

For calculating $C_{1212}$, the applied shear displacement is defined as:

$$
\begin{align*}
  u_1 &= \varepsilon_{12}x_2 \\
  u_2 &= \varepsilon_{12}x_1 \\
  u_3 &= 0
\end{align*}
$$

(14)

Note that in order to obtain a unit shear strain response in Abaqus, half value of unit shear strain should be specified for $\varepsilon_{12}$.

1.2.3.5 Calculating $C_{1111}$ constant and $C_{1212}$ constants
The constitutive equations relate stresses with strain as follows:

$$
\begin{bmatrix}
  \sigma_{11} \\
  \sigma_{22} \\
  \sigma_{33} \\
  \sigma_{23} \\
  \sigma_{31} \\
  \sigma_{12}
\end{bmatrix} = 
\begin{bmatrix}
  C_{1111} & C_{1122} & C_{1133} & C_{1123} & C_{1131} & C_{1112} \\
  C_{2211} & C_{2222} & C_{2233} & C_{2223} & C_{2231} & C_{2212} \\
  C_{3311} & C_{3322} & C_{3333} & C_{3323} & C_{3331} & C_{3312} \\
  C_{2311} & C_{2322} & C_{2333} & C_{2323} & C_{2331} & C_{2312} \\
  C_{3111} & C_{3122} & C_{3133} & C_{3123} & C_{3131} & C_{3112} \\
  C_{1211} & C_{1222} & C_{1233} & C_{1223} & C_{1231} & C_{1212}
\end{bmatrix} 
\begin{bmatrix}
  \varepsilon_{11} \\
  \varepsilon_{22} \\
  \varepsilon_{33} \\
  2\varepsilon_{23} \\
  2\varepsilon_{31} \\
  2\varepsilon_{12}
\end{bmatrix}
$$

(15)

Since only either uniaxial displacement or shear displacement is applied, the constitutive equations reduce to the following.

7
\[ \sigma_{11} = C_{1111}\varepsilon_{11} \]  \hspace{1cm} (16) \\
\[ \sigma_{12} = 2C_{1212}\varepsilon_{12} \]  \hspace{1cm} (17)

where \( \sigma_{11} \) is stress in x-direction, \( \sigma_{12} \) is shear stress in xy-plane, \( \varepsilon_{11} \) is strain in x-direction and \( \varepsilon_{12} \) is shear strain in xy-plane. \( W \), the energy of the system, is related to stress through equation 18 and 19. By incorporating equation 16 for uniaxial displacement case, and assuming the system experiences global displacement, \( C_{1111} \) can be found by equation 19 through 21. For shear displacement, \( C_{1212} \) can be found by equation 20 through 22.

\[ W = \frac{V}{2} \sigma_{y} \varepsilon_{y} \]  \hspace{1cm} (18) \\
\[ W = \frac{V}{2} C_{1111} \varepsilon_{11}^{2} \]  \hspace{1cm} (19) \\
\[ V = l_{x} l_{y} l_{z} \]  \hspace{1cm} (20) \\
\[ W = \sum_{i=1}^{N} e_{i} \]  \hspace{1cm} (21) \\
\[ W = 2V C_{1212} \varepsilon_{12}^{2} \]  \hspace{1cm} (22)

where \( V \) is the volume of the test box, \( e_{i} \) is the strain energy of a particular element, and \( N \) is the total number of elements in the system. All the information necessary to calculate \( C_{1111} \) and \( C_{1212} \) is obtained from the output file of Abaqus® postprocessing module. For comparison purpose, the value \( C_{1111} \) is normalized by \( C_{1111}^{\text{fiber}} \), which is the \( C_{1111} \) of linear elastic isotropic material having the elastic constants of the fiber and \( C_{1212} \) is normalized by \( C_{1212}^{\text{fiber}} \), which is the \( C_{1212} \) of linear elastic isotropic material having the elastic constants of the fiber. They can be calculated through the stress-strain relationship of such a material. \( C_{1111}^{\text{fiber}} \) is found through equations 23 and 24 [23].

\[ \sigma_{11} = \frac{E}{(1+\nu)(1-2\nu)} \left[ (1-\nu)\varepsilon_{11} + \nu(\varepsilon_{22} + \varepsilon_{33}) - (1+\nu)(\alpha\Delta T) \right] \]  \hspace{1cm} (23)

where \( E, \nu \) are elastic properties of fiber, \( \alpha \) is thermal expansion coefficient, \( \Delta T \) is change of temperature, and \( \varepsilon_{11}, \varepsilon_{22}, \varepsilon_{33} \) are strains in x, y, z-direction, respectively. Ignoring temperature effect, and after simplification using equation 16, we can obtain \( C_{1111}^{\text{fiber}} \) as follows:

\[ C_{1111}^{\text{fiber}} = \frac{E(1-\nu)}{(1+\nu)(1-2\nu)} \]  \hspace{1cm} (24)
\( C_{\text{fiber}}^{1212} \) is the same as the shear modulus of an isotropic material, which is given in equation 12.

1.3 RESULTS AND DISCUSSION

The fiber networks for both preferential and isotropic orientation are shown in Fig. 1.3(a) and 1.3(b). Figures 1.4(a) to 1.4(d) show the von Mises stress distribution, tension and compression stress, torsion stress distribution, and strain energy density distribution plots, respectively, for network with preferential fiber orientation while Fig. 1.4(e) to 1.4(h) show the contour plots for isotropically orientated fiber network. Notice that in Fig. 1.4(b) and Fig 1.4(f) some parts of the network experience compression stress, as shown by the negative value of the stresses. Therefore it is not suitable to use the current beam network model to model collagen network which has negligible compression stress. However, the current work can be extended to this system by inputting this nonlinear material property into Abaqus®. Besides, also note that for Fig. 1.4(c) and Fig 1.4(g), there are not much torsion stress since the beam network is under uni-directional displacement loading. Therefore the beam elements experience stress mostly from tension and compression.

We will investigate the effects of fiber orientation, void volume fraction and window size on \( C_{1111} \) before proceeding to \( C_{1212} \). Figure 1.5 shows the plot of normalized stiffness coefficient \( C_{1111} \) versus window size. Note that there is a decreasing exponential downward trend for normalized \( C_{1111} \) with increasing window size. It means that when the fibers lengths become shorter relative to the box that accommodates them, the normalized stiffness coefficient \( C_{1111} \) converges to a value. In other words, apparent property of normalized \( C_{1111} \) is approximated using the uniaxial displacement boundary condition. In fact, traction boundary condition should be performed along with the aforementioned displacement boundary condition to give upper and lower bound for the apparent property. However, there are several issues arise for using traction boundary condition. One of them is that the area connecting to the surface of the box is different for each element since the beams randomly oriented. Therefore the surface traction at the box surface for each beam will be hard to define. Another issue is that in order to use traction boundary condition, a constant force value will be applied at the box surface. This force will have to be divided among the beam elements touching the surface. Hence it will be problematic to prescribe value of forces at each end of the beams since each beam will sustain different loading. Nevertheless, the displacement boundary condition that is performed in this studies
confirm the trend that the response of the network converges to a certain value, which is approximated to a RVE, as the length scale of the fiber becomes smaller relative to the representative volume. Other phenomenon that can be observed includes less scattering of normalized $C_{1111}$ value as the window size increases. Again, this is due to the fact that the beam network approaches properties of a RVE when the window size becomes larger.

Figures 1.6 to 1.9 show the probability plots to fit the data for window sizes 1, 2, 3 and 5. There are up to 10 distribution functions under consideration: beta, gamma, Gumbel Min, Rayleigh, Weibull, $\chi$ and so on (Appendix A). We used Kolmogorov-Smirnov test to determine the top candidates for each four window sizes. The probability distribution that can fit the data the best is logistic distribution, followed by Gamma distribution and beta distribution. Note that the commonly used Weibull distribution does give a good fit to our data.

We will also like to investigate how different scales for an isotropically distributed beam network will affect the constitutive response, and compare them with that of beam network with preferential orientation. As can be observed from Fig. 1.10, the scattering trend for beam network with isotropic fiber orientation follows the trend of network with preferential fiber direction, which becomes narrower as the window size increases. In addition, there is a decreasing exponential downward trend for normalized $C_{1111}$ with increasing window size. All of these phenomena are to be expected since no matter how disoriented the network is, it will approach the RVE properties when the window size increases. One thing worth noting is that the constitutive response for isotropically distributed network is much lower than that of preferentially oriented network. Again, this is to be expected since preferentially oriented network can transfer the load much better than the disoriented network, therefore can sustain much higher loading and hence higher value for constitutive constant. Furthermore, the scattering in constitutive constant for the preferentially oriented fiber case is much higher than that of isotropic fiber network. This might indicate that the spatial heterogeneity in isotropic fiber network is smaller compared with preferentially oriented fiber network. Another observation that can be made during running the simulations is that, the elements removed in order to achieve certain value of volume fraction stays about the same for disoriented network while the elements removed for preferentially oriented network keeps increasing when the window size increases. Another observation that can be made on Fig. 1.5 and Fig. 1.10 is that, the normalized $C_{1111}$
values range for Fig. 1.10 is smaller than that of Fig. 1.10. In other words, the isotropic fiber network approaches the RVE response faster than that of preferentially oriented fiber network.

Besides window size studies, the effects of void volume fraction on the constitutive constant are investigated, as shown in Fig. 1.11 and 1.12, and Table 1.5 and 1.6. As expected, the constitutive response decreases with increasing void volume fraction. Furthermore, the constitutive responses do not cross the zero value of constitutive constant because our formulation will ensure all elements are connected to the clusters which can sustain load. Moreover, the scattering in constitutive values generally decreases with increasing void volume fraction. One phenomenon worth noting is that the network for smaller window size has steeper slope compared with that of larger window size. In other words, the constitutive response of the smaller window size should intercept the zero axes faster. One postulation is that the probability for the network with smaller window size to be disconnected is larger compared with that of larger window size. For instance, let the networks to be consisted of only three fibers and two of the fibers have to be broken in order to cause the network to fail. Let the networks under comparison to have window sizes 1 and 2, respectively. These networks have the same configuration, with only difference that the larger window size network has twice the number of elements of that of smaller window size network. In this case, network with larger window size has 6 elements while network with smaller window size has 3 elements. In this study, one larger element and two smaller elements are taken from the networks each time until the networks break. The network with larger window size has a probability of 0.2667 to be disconnected while network with smaller window size has a probability of 0.333. Since larger window size has more ways of element removal in order to avoid network disconnection, therefore it needs relatively more elements to be removed in order to result in failure.

Another study is conducted to investigate the relationship between degree orientation and void volume fraction of the network, as shown in Figs. 1.11 and 1.13, and Tables 1.5 and 1.7. As expected, the constitutive response for preferentially oriented fiber network is higher than that of isotropic fiber network. However, the preferentially oriented fiber network has a steeper slope. Using the analogy mentioned before, the preferentially oriented fiber network has relatively higher probability to be disconnected since there is less redundancy in the preferentially oriented network that can share the load when one element is removed.
The same studies have been performed to investigate the effect of fiber orientation, fiber volume fraction and window size on normalized $C_{1212}$. In each study, the same trend of normalized $C_{1111}$ and scattering effect can be observed on their counterparts, as shown from Table 1.8 though 1.12. These similarities for both studies on window size, fiber volume fraction and fiber orientation show that the same trend of constitutive response could be extended to other type of displacement loading. However, beam network with isotropic orientation can withstand the shear displacement better than the preferentially oriented beam network, as shown by comparing Table 1.8 and Table 1.9, and Table 1.10 and Table 1.12. Besides, the preferentially oriented fiber network approaches the RVE response faster than isotropic fiber network, by comparing Fig. 1.14 and Fig. 1.15.

Despite the differences, the percolation studies (Fig. 1.16 and Fig. 1.17) of $C_{1212}$ share the similarity of $C_{1111}$ in that, the network with smaller window size intercept the zero axes faster than that of larger windows. This shows that the composite with smaller window size, or smaller fiber in length scale, fails more rapidly than composite with larger window size when the fiber fails one by one. However, the slope of isotropic fiber network is relatively higher than the slope of preferentially oriented fiber network, which is the opposite of $C_{1111}$ case (Fig. 1.16 and Fig. 1.18). These two phenomena might suggest that when designing a fiber composite, the scattering angle between the fibers take precedence over length scale of the fiber, considering what kind of loading would be experienced by the composite.

In order to verify the removal of elements which does not sustain the stress, the strain energy density of the following two plots are compared. Figure 1.19(a) represents the network with all the dangling fibers and cluster while Fig. 1.19(b) represents the network without the dangling fibers. Note that the total strain energy for both networks is not the same. In fact, both networks should have the similar or almost similar amount of total strain energy since dangling fibers and clusters would not experience any loading and thus would have zero strain energy. Our purpose in this paper is to ensure a well connected fiber network in order to eliminate one variable due to these dangling elements on the volume fraction of the fiber. One check on the fiber network from our formulation confirms that there are no fibers that experience zero strain energy.

This leads to the various ways to define volume fraction in a network. When a network is generated, whether it is through manufacturing process as in an air filter case, or through
computer simulation as in our studies, we could always find those dangling fibers which do not contribute to load sharing but occupy a definite amount of spaces in the network. This will overestimate the volume fraction in the network, leading to incorrect representation of the load sharing capability of the network. In fact, there are three cases that can be used to define the volume fraction of a network, based on how we generate it. In this case, a volume fraction of 80 % is used, as shown in Table 1.13.

In Table 1.14, a case is being used to illustrate the implication of the above modeling methods for isotropic fiber network while in Table 1.15, a case is shown for preferentially oriented fiber network. In Table 1.14, normalized $C_{1111}$ value, volume fraction and percentage of elements removed does not vary significantly. This is to be expected since elements in isotropic fiber network are more easily connected to each other compared with elements in a preferentially oriented fiber network. Therefore, there are less dangling fibers leading to relatively constant values for parameters under investigation. While for Table 1.15, case II and case III have almost similar normalized $C_{1111}$ value, with discrepancy about 1.8 %, compared with a huge increase for case I. Note that case II is case III plus dangling fibers, and since dangling fibers do not carry any load, we should expect both cases to have similar normalized $C_{1111}$ value. As for case III, it has higher normalized $C_{1111}$ value since all the fibers are interconnected and contribute to the load sharing capability of the network. Besides, case III has lower volume fraction compared with other cases since approximately 16.83% of dangling fibers have been removed.

The above analysis poses three scenarios in which researchers have to pay attention when defining volume fraction of a network. Occasionally case II is being used in their formulation. However, the volume fraction might be overestimated since the actual volume fraction might be lower due to dangling fiber. In addition, the percentage of dangling fibers in the network is a variable that has to be taken into account if the network is to be generated isotropically. Therefore case I will be a more realistic representation of a network.

Other issue that needs to be considered is the effect of intersected volume due to how the volume fraction is calculated in this study:

$$v_f = \sum_{i=1}^{n} \pi r^2 l_i$$  \hspace{1cm} (25)

where $v_f$ is volume fraction, $r$ is the radius of fibers, and $l$ is the length for each fiber. Since this calculation does not take into account the intersected volume at each joint, the total volume
fraction of the fibers will be exaggerated and therefore underestimate the constitutive response. This effect will get more prominent when the fibers are aligned almost parallel to each others, as in this study. Besides that, when the window sizes get smaller, or the fibers get shorter compared with the length of the test box, the effect of intersected volume will be exaggerated as well. Therefore there is a need to calculate these intersected volumes in order to obtain more accurate result. Since the calculations to determine intersected volume are completed after this study, the related equations are placed in Appendix B.

Note that the numbers chosen for this study are not unique and they are served to show the relationship between the variables. In addition, the contribution from the micropolar component has been neglected to simplify the formulation. More studies can be extended from this study through some manipulations of the parameters. For example, nonlinear material behavior can be inputted into Abaqus® in order to fully capture the constitutive response of a collagen network, giving us the tool to model biological tissue such as single lamella in bone. Strength of the network can also be investigated through element removal at each realization after they have reached critical stress. Last but not least, a whole linear constitutive tensor could be found through a suitable application of loading, providing us the modeling framework for a fiber network.

1.4 CONCLUSIONS

We modeled a random fiber network as a beam network and predicted two stiffness components $C_{1111}$ and $C_{1212}$ as a function of several parameters that affect their magnitude and scatter. Through the finite element software, the beam network has been homogenized to give us the elastic constitutive response. The effect of dangling fibers was also investigated and was shown to play a crucial role in calculations especially for the preferentially oriented network.

1.5 REFERENCES


1.6 FIGURES AND TABLES

Table 1.1 Values of parameters used in generating fiber network.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Box dimension ($l_x* l_y* l_z$)</th>
<th>$W_{fiber}$</th>
<th>$T_{fiber}$</th>
<th>$V_{void}$ (%)</th>
<th>$\theta_{max}$ (°)</th>
<th>$\delta$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Values</td>
<td>1.5 * 1.5 * 0.15</td>
<td>0.05</td>
<td>0.05</td>
<td>10,20,40,60,80</td>
<td>10, 90</td>
<td>1,2,3,5</td>
</tr>
</tbody>
</table>

Table 1.2 Material properties and the type of boundary condition loading

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Elastic modulus (GPa)</th>
<th>Poisson ratio</th>
<th>$\epsilon_{11}$</th>
<th>$\epsilon_{12}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Values</td>
<td>1</td>
<td>0.3</td>
<td>1</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Table 1.3 Average values and standard deviation for normalized $C_{1111}$ for different window sizes for network with preferential fiber orientation and volume fraction 80 %.

<table>
<thead>
<tr>
<th>$\delta$</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{1111}/C_{fiber}$</td>
<td>0.279</td>
<td>0.124</td>
<td>0.0592</td>
<td>0.0236</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.0162</td>
<td>0.0168</td>
<td>0.0134</td>
<td>0.00576</td>
</tr>
</tbody>
</table>

Table 1.4 Average values and standard deviation for normalized $C_{1111}$ for different window sizes for network with isotropic fiber orientation and volume fraction 80 %.

<table>
<thead>
<tr>
<th>$\delta$</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{1111}/C_{fiber}$</td>
<td>0.111</td>
<td>0.0894</td>
<td>0.0638</td>
<td>0.0302</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.00998</td>
<td>0.00871</td>
<td>0.00426</td>
<td>0.00384</td>
</tr>
</tbody>
</table>

Table 1.5 Average values and standard deviation for normalized $C_{1111}$ for different void volume fractions for network with isotropic fiber orientation and $\delta = 2$.

<table>
<thead>
<tr>
<th>$V_{void}$ (%)</th>
<th>0.9</th>
<th>0.8</th>
<th>0.6</th>
<th>0.4</th>
<th>0.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{1111}/C_{fiber}$</td>
<td>0.00193</td>
<td>0.00691</td>
<td>0.0351</td>
<td>0.0638</td>
<td>0.0926</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.00104</td>
<td>0.00131</td>
<td>0.00543</td>
<td>0.00880</td>
<td>0.0117</td>
</tr>
</tbody>
</table>
Table 1.6 Average values and standard deviation for normalized $C_{1111}$ for different void volume fractions for network with isotropic fiber orientation and $\delta = 3$.

<table>
<thead>
<tr>
<th>$v_f^{\text{void}}$ (%)</th>
<th>0.9</th>
<th>0.8</th>
<th>0.6</th>
<th>0.4</th>
<th>0.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{1111}/C_{\text{fiber}}^{1111}$</td>
<td>0.00148</td>
<td>0.00309</td>
<td>0.0163</td>
<td>0.0403</td>
<td>0.0652</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.00114</td>
<td>0.000562</td>
<td>0.00276</td>
<td>0.00401</td>
<td>0.00378</td>
</tr>
</tbody>
</table>

Table 1.7 Average values and standard deviation for normalized $C_{1111}$ for different void volume fractions for network with preferential fiber orientation and $\delta = 2$.

<table>
<thead>
<tr>
<th>$v_f^{\text{void}}$ (%)</th>
<th>0.9</th>
<th>0.8</th>
<th>0.6</th>
<th>0.4</th>
<th>0.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{1111}/C_{\text{fiber}}^{1111}$</td>
<td>0.00959</td>
<td>0.0188</td>
<td>0.0516</td>
<td>0.0813</td>
<td>0.124</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.00377</td>
<td>0.00621</td>
<td>0.0128</td>
<td>0.0185</td>
<td>0.0202</td>
</tr>
</tbody>
</table>

Table 1.8 Average values and standard deviation for normalized $C_{1212}$ for different window sizes for network with preferential fiber orientation and volume fraction 80%.

<table>
<thead>
<tr>
<th>$\delta$</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{1212}/C_{\text{fiber}}^{1212}$</td>
<td>0.00933</td>
<td>0.00308</td>
<td>0.00102</td>
<td>0.000276</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.000578</td>
<td>0.000461</td>
<td>0.000444</td>
<td>0.0000985</td>
</tr>
</tbody>
</table>

Table 1.9 Average values and standard deviation for normalized $C_{1212}$ for different window sizes for network with isotropic fiber orientation and volume fraction 80%.

<table>
<thead>
<tr>
<th>$\delta$</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{1212}/C_{\text{fiber}}^{1212}$</td>
<td>0.149</td>
<td>0.110</td>
<td>0.0818</td>
<td>0.0382</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.00904</td>
<td>0.0102</td>
<td>0.00795</td>
<td>0.00484</td>
</tr>
</tbody>
</table>

Table 1.10 Average values and standard deviation for normalized $C_{1212}$ for different void volume fractions for network with isotropic fiber orientation and $\delta = 2$.

<table>
<thead>
<tr>
<th>$v_f^{\text{void}}$ (%)</th>
<th>0.9</th>
<th>0.8</th>
<th>0.6</th>
<th>0.4</th>
<th>0.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{1212}/C_{\text{fiber}}^{1212}$</td>
<td>0.00348</td>
<td>0.0113</td>
<td>0.0426</td>
<td>0.0798</td>
<td>0.110</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.00226</td>
<td>0.00337</td>
<td>0.00381</td>
<td>0.00572</td>
<td>0.0102</td>
</tr>
</tbody>
</table>
Table 1.11 Average values and standard deviation for normalized $C_{1212}$ for different void volume fractions for network with isotropic fiber orientation and $\delta = 3$.

<table>
<thead>
<tr>
<th>$v_{\text{void}}$ (%)</th>
<th>0.9</th>
<th>0.8</th>
<th>0.6</th>
<th>0.4</th>
<th>0.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{1212}/C_{\text{fiber}}$</td>
<td>0.000504</td>
<td>0.00243</td>
<td>0.0199</td>
<td>0.0486</td>
<td>0.0818</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.000838</td>
<td>0.000978</td>
<td>0.00303</td>
<td>0.00608</td>
<td>0.00795</td>
</tr>
</tbody>
</table>

Table 1.12 Average values and standard deviation for normalized $C_{1212}$ for different void volume fractions for network with preferential fiber orientation and $\delta = 2$.

<table>
<thead>
<tr>
<th>$v_{\text{void}}$ (%)</th>
<th>0.9</th>
<th>0.8</th>
<th>0.6</th>
<th>0.4</th>
<th>0.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{1212}/C_{\text{fiber}}$</td>
<td>0.000158</td>
<td>0.000319</td>
<td>0.000884</td>
<td>0.00169</td>
<td>0.00321</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.0000881</td>
<td>0.000154</td>
<td>0.000290</td>
<td>0.000241</td>
<td>0.000373</td>
</tr>
</tbody>
</table>

Table 1.13 Cases of how we can define the volume fraction of a network. Note that Case I is being used in our studies.

<table>
<thead>
<tr>
<th>Case I</th>
<th>Case II</th>
<th>Case III</th>
</tr>
</thead>
</table>
| 1. Generate the network until 80 %.  
2. Remove any dangling fibers.  
3. Add more fibers and repeat step 2 until it reaches 80 %. | 1. Generate the network until 80 %.  
2. Do not remove any dangling fiber. | 1. Generate the network until 80 %.  
2. Remove any dangling fibers.  
3. Do not add more fibers. |

Table 1.14 Three cases of modeling methods for isotropic fiber network.

<table>
<thead>
<tr>
<th>Cases</th>
<th>$C_{1111}/C_{\text{fiber}}$</th>
<th>Volume fraction (%)</th>
<th>Elements removed (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case I</td>
<td>0.0968</td>
<td>80</td>
<td>0.545</td>
</tr>
<tr>
<td>Case II</td>
<td>0.0966</td>
<td>80</td>
<td>0</td>
</tr>
<tr>
<td>Case III</td>
<td>0.0965</td>
<td>80</td>
<td>0.547</td>
</tr>
</tbody>
</table>
Table 1.15 Three cases of modeling methods for preferentially oriented fiber network.

<table>
<thead>
<tr>
<th>Cases</th>
<th>$C_{1111}/C_{\text{fiber}}$</th>
<th>Volume fraction (%)</th>
<th>Elements removed (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case I</td>
<td>0.125</td>
<td>80</td>
<td>8.96</td>
</tr>
<tr>
<td>Case II</td>
<td>0.0792</td>
<td>80</td>
<td>0</td>
</tr>
<tr>
<td>Case III</td>
<td>0.0778</td>
<td>62.6</td>
<td>16.83</td>
</tr>
</tbody>
</table>
Figure 1.1 Test box and regions of trimming when the other end of the fiber ends at the other boxes.

Figure 1.2 Beam orientations with vectors defining beam cross-section and beam axis. \( t \) is the vector tangent to the beam axis, \( n_1 \) and \( n_2 \) are the vectors of the cross section in 1- and 2-directions, respectively.
Figure 1.3 (a) Loading plot of a beam network with the preferential fiber orientation under a uniaxial displacement boundary condition. (b) Loading plot of a beam network with the isotropic fiber orientation under a uniaxial displacement boundary condition.
Figure 1.4 (a) Von Mises stress distribution plot of a beam network with fibers arranged in preferential fiber orientation. (b) Tension and compression stress distribution plot of beam network with preferential fiber orientation. (c) Torsion stress distribution plot of beam network with preferential fiber orientation. (d) Strain energy density distribution plot of beam network with preferential fiber orientation. (e) Von Mises stress distribution plot of a beam network with fibers isotropically arranged. (f) Tension and compression stress distribution plot of beam network with fibers isotropically arranged. (g) Torsion stress distribution plot of beam network
with fibers isotropically arranged. (h) Strain energy density distribution plot of beam network with fibers isotropically arranged.

Figure 1.5 Window size study of $C_{111}$ for beam network with preferential fiber orientation and volume fraction 80%, along with standard deviation bar.
Figure 1.6 Fits by several distribution functions for the case of $\delta = 1$.

Figure 1.7 Fits by several distribution functions for the case of $\delta = 2$. 
Figure 1.8 Fits by several distribution functions for the case of $\delta = 3$.

Figure 1.9 Fits by several distribution functions for the case of $\delta = 5$.  

Legend
- Data
- LS Beta
- LS Chi Dist.
- LS Gamma
- LS Gumbel Max
- LS Gumbel Min
- LS Logistic
- LS Normal
- LS Rayleigh
- LS Rectangular
- LS Weibull
Figure 1.10 Window size study of $C_{1111}$ for beam network with isotropic fiber orientation and volume fraction 80%, along with standard deviation bar.

Figure 1.11 Percolation study of $C_{1111}$ of beam network with isotropic fiber orientation and window size 2, along with standard deviation bar.
Figure 1.12 Percolation study of $C_{1111}$ of network with isotropic fiber orientation and window size 3, along with standard deviation bar.

Figure 1.13 Percolation study of $C_{1111}$ of network with preferential fiber orientation and window size 2, along with standard deviation bar.
Figure 1.14 Window size study of $C_{1212}$ for network with preferential fiber orientation and volume fraction 80%, along with standard deviation bar.

Figure 1.15 Window size study of $C_{1212}$ for network with isotropic fiber orientation and volume fraction 80%, along with standard deviation bar.
Figure 1.16 Percolation study of $C_{1212}$ for network with isotropic fiber orientation and window size 2, along with standard deviation bar.

Figure 1.17 Percolation study of $C_{1212}$ for network with isotropic fiber orientation and window size 3, along with standard deviation bar.
Figure 1.18 Percolation study of $C_{1212}$ for network with preferential fiber orientation and window size 2, along with standard deviation bar.
Figure 1.19(a) Beam network with all the dangling fibers. (b) Beam network without any dangling fibers.
CHAPTER 2

MODELING OF OSTEON AND INTERSTITIAL LAMELLA

The material in this chapter is in part published in the following paper:
Elham Hamed, YikHan Lee and Iwona Jasiuk (2010) “Multiscale modeling of cortical bone,”
*Acta Mechanica*, in press [1].
This chapter summarizes my contribution to the above referenced paper.

2.1 INTRODUCTION
Bone is a biological material with hierarchical structure. It consists of several length scales, ranging from nanoscale to the whole bone level, as shown on Fig. 2.1[2]. At nanoscale, bone can be considered as a multiphase composite material, consisting of water, hydroxyapatite crystals, collagen and non-collagenous proteins. The crystals have irregular plate-like structure and are located within and outside of collagen fibrils. At sub-microscale or a single lamella level, bone consists of lamellar structure, which is plate–like for secondary lamella and cylindrical for osteonal lamella. Within each lamella, the mineralized collagen fibers align themselves in a preferentially oriented direction. Sometimes the fibers will cross-over each other, resulting in a bond or cross-link that bound them together. At microscale or laminate level, the lamellas from the previous scale stack on each other in different orientations to form osteon and secondary lamella. The orientations include orthogonal (0/90°) structure, plywood structure [3], and helical structure [4]. Osteons, secondary lamella and resorption cavities then form the cortical bone at the mesoscale level. Macroscale level is whole bone, including cortical and trabecular bone, or organ level. This chapter will focus on modeling bone at microscale level.

2.2 MODELING AT MICROSTRUCTURAL LEVEL
At this level, one can distinguish two lamellar structures in mature cortical bone, which are osteon and interstitial lamella.

2.2.1 Modeling of a single osteon
Effective elastic properties of a single osteon are calculated using a generalized-self consistent method (GSC) following the approach of Dong and Guo [5]. At this level, the osteon is modeled as a two phase composite with the osteonal lamella being a matrix and the Haversian canal being a hollow cylindrical inclusion. The properties of osteonal lamella, used as inputs in this model,
are transversely isotropic. These combined with the aligned hollow Harvesian canal result in the effectively transversely isotropic properties of the osteon. Thus, five effective elastic constants characterize an osteon.

In histological analyses the laminations appear as alternating light and dark layers under polarized light which are due to different orientations of collagen fibrils within the adjacent lamellae [6]. The axis between the adjacent layers can differ by as much as 90 degrees. Several types of fibril orientations in osteonal lamellae were reported in literature. They include orthogonal and twisted plywood models [3] with the twisted plywood motif being most common. In this orientation pattern, there is a fixed angle of orientation between each lamella and there is a rotation along the longitudinal axis, Fig. 2.2 [7].

In this chapter, we model the elastic properties of osteonal lamella following the homogenization scheme of Sun and Li’s [8] developed for laminated composite materials. In their model, Sun and Li divided a thick laminate into large numbers of repeating sublaminates. Each sublamine was treated as a three-dimensional homogeneous anisotropic solid which served as a representative volume element for the whole laminate. Sublaminates were, in turn, composed of several laminas (lamellae) with different stacking of fibril orientation. The thickness of a typical sublaminar was assumed to be small compared with that of the whole laminate. In addition, the in-plane dimensions were kept infinitesimal so that the stresses and strains in each lamina were uniform in the planar directions. From the consideration of traction and displacement continuum conditions at the interfaces of the laminas, Sun and Li further assumed constant in-plane stresses in the $x_1x_2$ plane, and constant out-of-plane strains, through $x_3$ axis, as follows

\[
\begin{align*}
\varepsilon^{(k)}_{11} &= \varepsilon_{11}, & \sigma^{(k)}_{33} &= \sigma_{33}, \\
\varepsilon^{(k)}_{22} &= \varepsilon_{22}, & \sigma^{(k)}_{23} &= \sigma_{23}, \\
\varepsilon^{(k)}_{12} &= \varepsilon_{12}, & \sigma^{(k)}_{31} &= \sigma_{31},
\end{align*}
\]

where $\sigma^{(k)}_{ij}$ and $\varepsilon^{(k)}_{ij}$ are the stresses and strains in the $k^{th}$ lamina. Next, we use the contracted notation for stress $[\sigma_1, \sigma_2, \sigma_3, \sigma_4, \sigma_5, \sigma_6]^T = [\sigma_{11}, \sigma_{22}, \sigma_{33}, \sigma_{23}, \sigma_{13}, \sigma_{12}]^T$ and strain $[\varepsilon_1, \varepsilon_2, \varepsilon_3, \varepsilon_4, \varepsilon_5, \varepsilon_6]^T = [\varepsilon_{11}, \varepsilon_{22}, \varepsilon_{33}, \varepsilon_{23}, \varepsilon_{13}, \varepsilon_{12}]^T$. Then, the stress-strain relationship for a laminate, with the $x_1x_2$ plane being the plane of symmetry, is expressed as
The effective elastic constants of the sublaminate, $\tilde{C}_{ij}$, can be defined in terms of elastic properties of laminas (lamellae) as

\[
\begin{align*}
\tilde{C}_{11} &= \sum_{k=1}^{N} \Phi_k C_{11}^{(k)} + \sum_{k=2}^{N} (C_{13}^{(k)} - \lambda_{13}) \Phi_k (C_{13}^{(1)} - C_{13}^{(k)}) / C_{33}, \\
\tilde{C}_{12} &= \sum_{k=1}^{N} \Phi_k C_{12}^{(k)} + \sum_{k=2}^{N} (C_{13}^{(k)} - \lambda_{13}) \Phi_k (C_{23}^{(1)} - C_{23}^{(k)}) / C_{33}, \\
\tilde{C}_{13} &= \sum_{k=1}^{N} \Phi_k C_{13}^{(k)} + \sum_{k=2}^{N} (C_{33}^{(k)} - \lambda_{33}) \Phi_k (C_{13}^{(1)} - C_{13}^{(k)}) / C_{33}, \\
\tilde{C}_{22} &= \sum_{k=1}^{N} \Phi_k C_{22}^{(k)} + \sum_{k=2}^{N} (C_{23}^{(k)} - \lambda_{23}) \Phi_k (C_{23}^{(1)} - C_{23}^{(k)}) / C_{33}, \\
\tilde{C}_{23} &= \sum_{k=1}^{N} \Phi_k C_{23}^{(k)} + \sum_{k=2}^{N} (C_{33}^{(k)} - \lambda_{33}) \Phi_k (C_{23}^{(1)} - C_{23}^{(k)}) / C_{33}, \\
\tilde{C}_{33} &= 1 / \sum_{k=1}^{N} \Phi_k / C_{33}^{(k)}, \\
\tilde{C}_{16} &= \sum_{k=1}^{N} \Phi_k C_{16}^{(k)} + \sum_{k=2}^{N} (C_{13}^{(k)} - \lambda_{13}) \Phi_k (C_{36}^{(1)} - C_{36}^{(k)}) / C_{33}, \\
\tilde{C}_{26} &= \sum_{k=1}^{N} \Phi_k C_{26}^{(k)} + \sum_{k=2}^{N} (C_{23}^{(k)} - \lambda_{23}) \Phi_k (C_{36}^{(1)} - C_{36}^{(k)}) / C_{33},
\end{align*}
\]
\[\overline{C}_{36} = \sum_{k=1}^{N} \Phi_k C_{36}^{(k)} + \sum_{k=2}^{N} (C_{33}^{(k)} - \lambda_{33}) \Phi_k (C_{36}^{(k)} - C_{36}^{(k)}) / C_{33}^{(k)},\]

\[\overline{C}_{66} = \sum_{k=1}^{N} \Phi_k C_{66}^{(k)} + \sum_{k=2}^{N} (C_{36}^{(k)} - \lambda_{36}) \Phi_k (C_{36}^{(k)} - C_{36}^{(k)}) / C_{33}^{(k)},\]

\[\overline{C}_{44} = \left( \sum_{k=1}^{N} \Phi_k C_{44}^{(k)} / \Delta_k \right) / \Delta,\]

\[\overline{C}_{45} = \left( \sum_{k=1}^{N} \Phi_k C_{45}^{(k)} / \Delta_k \right) / \Delta,\]

\[\overline{C}_{55} = \left( \sum_{k=1}^{N} \Phi_k C_{55}^{(k)} / \Delta_k \right) / \Delta,\]

with

\[\lambda_{13} = \overline{C}_{13}, \quad \lambda_{23} = \overline{C}_{23}, \quad \lambda_{33} = \overline{C}_{33}, \quad \lambda_{36} = \overline{C}_{36},\]

\[\Delta = \left( \sum_{k=1}^{N} \Phi_k C_{44}^{(k)} / \Delta_k \right) \left( \sum_{k=1}^{N} \Phi_k C_{55}^{(k)} / \Delta_k \right) - \left( \sum_{k=1}^{N} \Phi_k C_{45}^{(k)} / \Delta_k \right)^2,\]

\[\Delta_k = C_{44}^{(k)} C_{55}^{(k)} - (C_{45}^{(k)})^2,\]

where \(C_{ij}^{(k)}\) is the elastic stiffness of the \(k^{th}\) single lamina with its own specific fibril orientation and \(N\) shows the number of laminas of arbitrary thickness within a sublaminate. \(v_k\) is the volume fraction of the \(k^{th}\) lamina given by \(v_k = t_k / h\) with \(t_k\) being the thickness of the \(k^{th}\) lamina and \(h\) the total thickness of the sublaminate. Since each single lamina has a different fibril orientation, a transformation matrix is needed to rotate the fibril angle in each lamina. The transformation matrix, \(T_{ij}\), is defined as [9]

\[
[T_{ij}] = \begin{bmatrix}
  m_1^2 & n_1^2 & p_1^2 & 2n_1p_1 & 2p_1m_1 & 2m_1n_1 \\
  m_2^2 & n_2^2 & p_2^2 & 2n_2p_2 & 2p_2m_2 & 2m_2n_2 \\
  m_3^2 & n_3^2 & p_3^2 & 2n_3p_3 & 2p_3m_3 & 2m_3n_3 \\
  m_1m_3 & n_1n_3 & p_1p_3 & n_1p_3 + n_3p_1 & p_1m_3 + p_3m_1 & m_1n_3 + m_3n_1 \\
  m_1n_1 & n_1p_1 & p_1n_1 + n_1p_3 & p_1m_1 + p_3m_3 & m_1n_1 + m_1n_3 \\
  m_1n_2 & n_1n_2 & p_1p_2 & n_1p_2 + n_2p_1 & p_1m_2 + p_2m_1 & m_1n_2 + m_2n_1
\end{bmatrix},
\]

where \(m_i, n_i,\) and \(p_i\) are the direction cosines of the axis \(i (i = 1, 2, 3),\) that is

\[m_1 = \cos \theta_{x1}, \quad n_1 = \cos \theta_{y1}, \quad p_1 = \cos \theta_{z1},\]
\[ m_2 = \cos \theta_2, \quad n_2 = \cos \theta_2, \quad p_2 = \cos \theta_2, \]
\[ m_3 = \cos \theta_3, \quad n_3 = \cos \theta_3, \quad p_3 = \cos \theta_3. \quad (7) \]

The \( 123 \) coordinate system is the sublaminate global Cartesian coordinate system, while the \( xyz \) coordinate system represents the local coordinate system of each lamina. The angles \( \theta_j \) are measured from the axis \( i \) to the axis \( j \), as shown in Fig. 2.3. After transformation, the stiffness tensor of the \( k^{th} \) lamina is obtained as
\[ C^{(k)} = T^{-1(k)} C T^{(k)}, \quad (8) \]
where \( C \) is the stiffness tensor of a single lamina. The effective stiffness tensor of the laminate is computed using Eqs. (4) and (5), and it is inverted to obtain the laminate effective compliance tensor \( \left[ \bar{S} \right] = \left[ \bar{C} \right]^{-1} \). Lastly, the effective engineering constants of the laminate are obtained
\[ \bar{E}_1 = \frac{1}{S_{11}}, \quad \bar{E}_2 = \frac{1}{S_{22}}, \quad \bar{E}_3 = \frac{1}{S_{33}}, \]
\[ \bar{v}_{23} = -\frac{S_{33}}{S_{22}}, \quad \bar{v}_{31} = -\frac{S_{31}}{S_{11}}, \quad \bar{v}_{12} = -\frac{S_{21}}{S_{11}}, \]
\[ \bar{G}_{23} = \frac{1}{S_{44}}, \quad \bar{G}_{31} = \frac{1}{S_{55}}, \quad \bar{G}_{12} = \frac{1}{S_{66}}. \quad (9) \]

Note that the effective moduli given above could not be obtained if the coupling coefficients \( \bar{C}_{16}, \bar{C}_{26}, \) and \( \bar{C}_{36} \) were not negligible.

In our problem, the osteonal lamella plays the role of a thick laminate and is broken into similar pieces called sublaminates. Every sublaminate consists of several single lamellae with twisted plywood pattern of collagen fibrils. Such a microstructure justifies the application of Sun and Li formulation [8] for the modeling of the osteonal lamella. This modeling procedure leads to a transversely isotropic response about the axis perpendicular to an osteon axis, which is placed along the axis \( x_1 \). A large number of sublaminates at different orientations about the osteon axis are then joined together into a ring forming the osteonal lamella, resulting in a transversely isotropic behavior along the osteon axis. Therefore, Sun and Li’s formulation is applied twice in modeling of the osteonal lamella. First, several lamellae, with the properties taken from the previous scale, the single lamella level, are rotated about the \( x_j \)-axis to find the effective properties of a sublaminate. Then, in the second step, several sublaminates are rotated
about the $x_f$-axis to obtain the effective elastic properties of the osteonal lamella. Figure 2.4 illustrates the microstructure of the osteonal lamella and the pertinent steps taken in its modeling.

2.2.2 Modeling of interstitial lamella
The evaluation of the effective elastic moduli of the interstitial lamella follows the same homogenization procedure as for the osteonal lamella case [8]. Homogenization at this level results in a transversely isotropic response along the bone axis. The interstitial lamella always arranges itself according to the preferential orientation of fibrils in the plane whose axis is parallel to the bone axis. Therefore, if a large number of such sublaminates are taken into account, the response will be transversely isotropic along the bone axis. In other words, these sublaminates can be treated as broken interstitial lamella rings. The difference between the broken osteonal lamella and interstitial lamella rings is that the ring pieces of the latter group are located between osteons while the osteonal lamella rings remain intact. This phenomenon can be viewed more vividly in Fig. 2.5. Another issue to be considered is the degree of mineralization. In general, the osteons are less stiff and less mineralized than the interstitial bone tissue [10,11]. Hence, we propose to use a higher degree of mineralization for an interstitial lamella as compared to an osteon.

2.3 MODEL INPUTS AND PARAMETERS
2.3.1 Single osteon
A typical osteon is a cylinder about 250 µm in diameter and approximately 1 cm long, while the diameter of Haversian canal is approximately 50 µm [6]. This gives the volume fraction of the canal of about 4%. Within the central canal run blood vessels, lymphatics, nerves and connective tissues that continue through the bone marrow and periosteum [6]. Therefore, the mechanical properties of the Haversian canal can be assumed to be similar to that of water. As discussed earlier, the degree of mineralization is higher in the interstitial lamella than in the osteon. Therefore, we assume the highest mineral volume fraction, i.e. 50%, for the interstitial lamella in the fully-mineralized mature bone, whereas the degree of mineralization in the osteon is taken to be 42%, the intermediate value between the upper and lower bounds. The properties of the osteonal lamella are calculated using the results for the single lamella for the mineral volume fraction of 42%, which is given as
More detailed information regarding single lamella calculation can be obtained from paper [2]. For simplification purpose, Mori-Tanaka method, which treats mineralized fibrils as cylindrical inclusion while mineral crystals hydroxy-apatite as matrix, is used to obtain the values in Eqs. (10).

Giraud-Guille observed twisted plywood architecture of collagen fibrils for human osteons [7]. Hence, here we adopt the assumption of the twisted plywood motif for fibril orientation. The starting angle, the angle which gives the largest elastic modulus, is chosen to be a 0 degree for the innermost layer. The phenomenon of decreasing elastic modulus from the innermost layer was confirmed by nanoindentation experiments [12]. Since the osteon does not have a fixed number of osteonal lamella layers and, to our knowledge, there is no data available in the literature about the orientation of the osteon’s outermost layer, we assume that the fibrils complete a 180 degrees turn from the innermost to the outermost layer. This will result in an anti-symmetric laminate with the in-plane isotropy. As long as the layers are not orthogonal to each other, the angle change between successive layers has a negligible effect on the results [13]. Another parameter to consider is the degree of mineralization of the osteonal lamella. For simplicity, we assume that the DOM is not changing for different layers.

2.3.2 Interstitial lamella
As mentioned earlier, the DOM in the interstitial lamella is greater than that of the osteons. Hence, we select the degree of mineralization of the interstitial lamella to be 50%.
2.4 RESULTS

2.4.1 Single Osteon

Using the mentioned parameters, the elastic stiffness tensor of osteonal lamella, $C_{\text{osteonal lamella}}$, is obtained as

$$
C_{\text{osteonal lamella}} = \begin{bmatrix}
20.16 & 4.01 & 4.01 & 0 & 0 & 0 \\
4.01 & 14.12 & 4.33 & 0 & 0 & 0 \\
4.01 & 4.33 & 14.12 & 0 & 0 & 0 \\
0 & 0 & 0 & 5.13 & 0 & 0 \\
0 & 0 & 0 & 0 & 6.38 & 0 \\
0 & 0 & 0 & 0 & 0 & 6.38
\end{bmatrix} \text{ GPa.} \tag{12}
$$

Using the generalized-self consistent method with the osteonal lamella being the matrix and the Haversian canal being the inclusion (inhomogeneity), the transversely isotropic elastic constants of a single osteon, $C_{\text{osteon}}$, are calculated to be

$$
C_{\text{osteon}} = \begin{bmatrix}
19.46 & 3.93 & 3.93 & 0 & 0 & 0 \\
3.93 & 13.15 & 4.51 & 0 & 0 & 0 \\
3.93 & 4.51 & 13.15 & 0 & 0 & 0 \\
0 & 0 & 0 & 4.32 & 0 & 0 \\
0 & 0 & 0 & 0 & 5.82 & 0 \\
0 & 0 & 0 & 0 & 0 & 5.82
\end{bmatrix} \text{ GPa.} \tag{13}
$$

2.4.2 Interstitial Lamella

Following the method described in Section 2.2, the stiffness tensor of the interstitial lamella is calculated to be

$$
C_{\text{interstitial}} = \begin{bmatrix}
23.88 & 4.69 & 4.69 & 0 & 0 & 0 \\
4.69 & 16.53 & 5.08 & 0 & 0 & 0 \\
4.69 & 5.08 & 16.53 & 0 & 0 & 0 \\
0 & 0 & 0 & 6.01 & 0 & 0 \\
0 & 0 & 0 & 0 & 7.51 & 0 \\
0 & 0 & 0 & 0 & 0 & 7.51
\end{bmatrix} \text{ GPa.} \tag{14}
$$

2.5 DISCUSSION

Tables 2.1 and 2.2 show, respectively, the longitudinal and transverse elastic moduli of the osteonal lamella, the single osteon, the interstitial lamella, and the cortical bone obtained by
using our model. These tables also give the selected experimental data available in the literature to allow a comparison with our results. Our analytical results are in a reasonably good agreement with experiments. It should be noted that since it is difficult to distinguish the osteonal lamella from the interstitial lamella in the transverse direction, not much data is available in the literature on the transverse elastic moduli of those lamellae and, instead, most of the works report the average elastic modulus of cortical bone in the transverse direction.

We have made simplifying assumptions in order to derive our model. One issue to be considered is the fibril orientation in different lamellas at the osteon level. Here, we adopted the twisted plywood pattern, whereas other patterns, like orthogonal plywood motif, were also observed. The orthogonal plywood model consists of collagen fibrils which are parallel in a given plane but, unlike the twisted plywood fibrils, do not rotate continuously from plane to plane. Instead, the fibrils can only take on one of two directions which are out of phase 90° with each other. Figure 2.6 shows TEM images of both orthogonal and twisted plywood structures [14]. The orthogonal and twisted plywood models predict different elastic symmetries for the osteon and the whole bone: orthogonal plywood has an orthotropic elastic symmetry while the twisted plywood does not [15]. Even if we choose the twisted plywood architecture of collagen fibrils as it was assumed in many previous works [3, 16], there might not be a fixed degree of rotation between each layer of the lamella. For instance, Weiner [17] found that there is a bimodal peak at 30° and 70° in the collagen fibril arrays suggesting that the angle changes in increments of 30° from 0° to 120° and then back to 0° again. In other words, there is a discontinuity between lamellas at 120° and 0°. If the lamella does not complete the turning of 180°, an in-plane isotropic response could not be obtained. All together these would introduce some anisotropy into the laminated lamella structure, therefore skewing the results. Another phenomenon captured by experiments is that the degree of mineralization decreases from the interior part of the osteon to the middle part and then increases again until reaching the cement line [18]. Hence, DOM has a gradient instead of a constant value, as assumed here. Besides that, the bone area corresponding to the lowest degree of mineralization is 50 to 60% less mineralized than the area in which bone is fully, if not completely, mineralized [19]. Since the interstitial lamella is known to have higher DOM than the osteonal lamella, if the interstitial lamella is taken to be the fully mineralized region, the trough of DOM changes in osteon should be half of
that of the interstitial lamella. Therefore, the DOM value assumed in our model might be higher than in the actual case.

At this level, we tried to select a model capable of capturing the microstructure and physics of the corresponding scale. Yet, those selections are not unique and other modeling techniques could be applied alternatively. As an example, here we used Sun and Li formulation [8] to model the osteonal and interstitial lamella while we initially tried the classical laminate theory to describe the elastic behavior of these lamellae. According to the classical laminate theory, the overall behavior of the multidirectional laminate is a function of the material properties and the stacking orientation of the individual layers [9]. The behavior of the laminate is predicted using several assumptions such as the Kirchoff hypothesis that all planes remain plane, the plane stress state, the perfect bonding between each layer of fibrils, the continuous displacement throughout the laminate, and the linear stress-strain relationship. The formulation for using this approach is included in the Appendix C. However, this approach was not finally selected since the plane stress assumption makes it difficult to evaluate the properties through the thickness. For the complete turning of plywood motif, the in-plane isotropic laminate could be obtained which after turning around another axis, in order to simulate the broken pieces of lamella ring, resulted in an isotropic response instead of the transversely isotropic one that we obtained using the Sun and Li model described in this paper. This approach also yielded relatively lower values of 15 GPa and 17.8 GPa for the elastic modulus of the osteonal lamella and the interstitial lamella, respectively, as compared with the current values of 18.52 GPa and 21.74 GPa. Hence, we came to the conclusion that Sun and Li’s formulation [7] is more appropriate since it could model the through-thickness behavior and the laminates could have different in-plane and through-thickness properties, resulting in the transversely isotropic interstitial lamella and osteonal lamella along the osteon axis. In future work, the stiffness coefficients found in Chapter 1 can serve as the input for this scale. Parametric studies on fiber orientation and degree of mineralization can also be performed in order to correlate their influences on the mechanical properties of osteon and interstitial lamella.

2.6 CONCLUSIONS
In this study, Sun & Li homogenization scheme was used to model osteonal lamella and interstitial lamella while general-self consistent method was used to model osteon. Several assumptions had been made, such as constant degree of mineralization across lamellas, plywood
structure of fiber orientation, and transversely isotropic geometry of osteon and interstitial lamella. Overall, the results were in good comparison with other analytical models and experimental data.

2.7 REFERENCES


### 2.8 FIGURES AND TABLES

Table 2.1 Comparison of present results for the longitudinal elastic moduli with experiments.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Longitudinal elastic modulus (GPa)</th>
<th>Experimental data</th>
<th>Bone type</th>
<th>Testing technique</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Our model</td>
<td>Measurements</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osteonal lamella</td>
<td>18.52</td>
<td>22.5±1.3 (1997) [20]</td>
<td>Human tibia</td>
<td>Nanoindentation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15.8±5.3 (1999) [21]</td>
<td>Human neck</td>
<td>Nanoindentation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>17.8±1.7 (2002) [22]</td>
<td>Human tibia</td>
<td>Nanoindentation</td>
</tr>
<tr>
<td>Interstitial lamella</td>
<td>21.74</td>
<td>25.8±0.7 (1997) [20]</td>
<td>Human tibia</td>
<td>Nanoindentation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>17.5±5.3 (1999) [21]</td>
<td>Human neck</td>
<td>Nanoindentation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20.1±1.7 (2002) [22]</td>
<td>Human tibia</td>
<td>Nanoindentation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22±3 (2005) [26]</td>
<td>Human femur</td>
<td>Nanoindentation</td>
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<td></td>
<td></td>
<td>33.8±7.5 (2006) [23]</td>
<td>Human radius</td>
<td>SR-µCT a</td>
</tr>
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</table>

*a Synchroton radiation micro computed tomography*
Table 2.2 Comparison of present results for the transverse elastic moduli with experiments.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Transverse elastic modulus (GPa)</th>
<th>Experimental data</th>
<th>Measurements</th>
<th>Bone type</th>
<th>Testing technique</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Our model</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Osteon</strong></td>
<td>11.24</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.1 Hierarchical structure of bone. (a) cortical and cancellous bone; (b) osteons with Haversian systems; (c) lamella; (d) collagen assemblies of collagen fibrils; (e) bone mineral crystals, collagen molecules, and non-collagenous proteins [2].

Figure 2.2 Twisted plywood patterns of fibrils in osteon [7].
Figure 2.3 Illustration of the global and local coordinate systems referred to in the three-dimensional transformational relations.
Figure 2.4  (a) (side view) Single lamella containing fibrils in a preferential direction, (b) (side view) Several single lamella with different fibril orientations stack together in a twisted plywood pattern to form the sublaminate, which is transversely isotropic in direction perpendicular to the osteon axis, (c) (sky view) Several sublaminates can be arranged to form a ring resembling osteonal lamella. Note that this arrangement results in transversely isotropic properties along osteon axis (d) (sky view) Osteonal lamella surrounding the Haversian canal in an osteon.
Figure 2.5 Cortical bone comprised of osteon and interstitial lamella. Osteonal lamellas make a complete circle while interstitial lamellas form, approximately, rectangular shape in between osteonal lamellas. Note that interstitial lamella can be envisioned as broken ring pieces that encircle the osteon. Taking into account a huge number of such rings encircling an osteon would result in transversely isotropic response for interstitial lamella.
Figure 2.6 TEM micrographs showing different fibril orientation patterns within neighboring lamellae: (a) orthogonal plywood motif; successive layers of alternating longitudinally (L) and transversely (T) sectioned fibrils are evident and (b) twisted plywood motif; a successive transition of longitudinally (L), obliquely (O) and transversely sectioned (T) fibrils is apparent [14].
CHAPTER 3
ULTRASOUND SCANNING AND CHARACTERIZATION OF BONE GROWTH

3.1 INTRODUCTION
This is an interdisciplinary project bridging several disciplines spanning from biology to engineering. It involves four UIUC faculty: Dr. Iwona Jasiuk and Dr. Nicholas Fang from Mechanical Science and Engineering, Dr. William O’Brien from Electrical and Computer Engineering, Director of Bioacoustics Research Laboratory (all three being members of Beckman Institute) and Dr. Jo Ann Cameron from Cell and Developmental Biology. The objective of ultrasonic scanning is to detect the presence of cartilage tissue which will then calcify to become bone. The challenges facing here is the difficulty in identifying border between bone and cartilage. In order to determine the regions of bone, cartilage and soft tissue, a specially written program which gives the backscatter coefficient (BSC) at a certain frequency is used here. Ultrasonic waves are particularly affected by obstacles and inhomogeneities, such as discontinuity in density or elasticity [1]. Small obstacles give rise to a scattered wave radiated in all directions. BSC is used to measure scattering from biological tissue since it is related to medium characteristic.

3.2 BACKGROUND
Understanding how to regenerate human tissues which normally heal by scarring following injury [2, 3] is a major challenge in regenerative medicine field. In humans and other vertebrates bone fractures can heal to restore structural and functional integrity. However, when an injury produces a large gap in bone, the fibrous scar tissue instead of bone will fill in the gap. In this project, the adult frog (Xenopus laevis) is selected as an animal model because Xenopus tadpoles have the ability to regenerate limb structures, but lose this ability as they mature. Thus, clues from limb development and successful regeneration in Xenopus tadpoles will be used as guides to achieve complete large size bone defect repair in adult Xenopus hind limbs through recapitulation of normal long bone development. Specifically, an experimentally damaged limb will be induced to generate cartilage template within a missing bone bed which will then ossify.
Traditional approaches of characterizing the developmental growth of ossified tissue require euthanizing the animal. These include histology, transmission and scanning electron microscopy, and micro-CT. These also do not allow the possibility of a follow-up study on the same animal. Since every biological individual is unique, i.e. the condition and recovery rate are slightly different, the results will vary from sample to sample. Thus, there are major advantages in using a non-invasive technology such as the ultrasound to study the growth of ossified tissue in vivo. Ultrasonic technique have been used to provide reliable information on assessment of fetal growth [4], to estimate bone quality [5], to characterize solid tumor [6], and for other applications. However, to our knowledge, the ultrasound technique has not been used in tissue engineering studies. Ultrasonic measurements can give bone’s modulus measurements which can be correlated with bone strength. Thus, the ultrasounds’ predictive ability is similar to that of X-ray densitometry measurement of bone mineral content [7]. This study serves as the pre-cursor to the future research of using ultrasound to characterize non-invasively, the progress of the cartilage-to-bone repair from the initial stages of repair through ossification.

3.3 METHODOLOGY

3.3.1 Sample Preparation

A large portion of the tarsus bone was removed from the frog hind limb (Figure 3.1) and a novel biocompatible scaffold that contained and released growth factors was inserted. These would promote de novo cartilage-to-bone development within this critical bone defect space. After three months, the hind leg of the frog with surgery on it was harvested for ultrasound scanning.

3.3.2 Scanning procedure

Hind limb sample was prepared into size of interest and any dangling soft tissue was removed. Tank for ultrasonic testing was filled with degassed water. Rubber was then placed at the bottom of the tank to provide friction to the sample so that it will not drift away during scanning. The setup was as shown in Fig. 3.2. Transducer with suitable frequency was chosen. Higher frequency can provide better resolution but at a cost of higher attenuation, especially to a bone sample which has higher impedance compared with soft tissue. Lower frequency can avoid attenuation issue but at a lower resolution and higher imaging depth. Another aspect to take into account is the resolution of the scanning. The resolution of the scanning was made sure to be
smaller than the length scale of the tissue or region to be scanned. The resolution is estimated to be the same as the wave length of the sound wave in water, which is as follows:

\[ \lambda = \frac{SOS}{f} \]

In this experiment, transducer with frequency of 20 MHz was chosen. Transducer was connected to a connector which was then connected to a BNC to BNC plug. The combination was inserted into a rubber holder which then fixed to a clamp. BNC to BNC plug was used to connect the connector and to the T/R of the Computer Controlled Pulse/Receiver Model 5900 PR machine (Daedal 5800 machine would be used if transducer had low frequency, which was around 1 MHz). The clamp was then attached to the ultrasonic system. Plexiglass (PMMA) was placed on the rubber in the water tank under the transducer to provide a reference spectrum. 2 BNC cables were used to connect “input 1” and “trigger in” from PDA14 scope to “receiver” and “sync” from 5900 machine, respectively. Shorter cable was used to connect input to receiver to reduce signal noise. 5900 machine was turned on and the program Daedal Menu was opened. After that, the sub-program on the menu, named PDA14 was opened. This program illustrates the strength of signal across the sample. The configuration on 5900 machine for 20 MHz transducer was set in this step. Mode was chosen to be P/E which means pulse/echo. Energy was chosen to be 4 micro joules first and was calibrated in a later step. Low pass filter was set to be around 50 MHz to filter any frequency higher than the chosen frequency. Program named position on Daedal Menu was opened. The objective of this step was to determine the focus of the transducer and to obtain signal around axial focus. The transducer was lowered until a good spectrum could be obtained at the focus position. With this focus position, settings on the PR 5900 were adjusted. Care had to be taken so that the transducer face was parallel to the sample face, and avoided any bubble under transducer face. Noise on the scope could be reduced (which translated to clearer picture of the signal) by enabling sweeping option in the program. However, the function was only turned on after the transducer has finished moving. In this case, range of 25 to 75 was chosen, where higher number could give better sweeping but took longer time. When a clear signal could not be obtained, the window size of the scope and the voltage range of the input signal were adjusted. Power spectrum in the program was chosen after the region of signal was selected. The purpose of this step was to check whether transducer was overdrove, which happened when energy above optimum level was put into transducer. Higher energy was
used when the transducer has low frequency. In this case, the scope was zoomed in into region in the power spectrum which gave strongest amplitude, which occurs normally at around the transducer frequency. If the center of the highest amplitude was ahead of the transducer frequency, the energy input would be reduced and vice versa. Since a smooth instead of a curly spectrum was desired, attenuation decibel would be increased and energy level would be decreased when the spectrum was far from optimum. However, a combination of higher energy and lower attenuation was desired. Therefore the transducer can be varied until 10 to 15 percents of its center frequency. Besides that, the frequency band of the power spectrum had to be checked so that it is smooth. Taking all those factors into consideration, 8 µJ of energy and 0 dB attenuation were chosen.

Next, the reference spectrum was ready to be scanned. “2D” option in the main menu was opened. The first axis was set to 3 so that transducer moved in the z direction (scanning in z direction). Suitable distance and step size were chosen, depending on the thickness of the glass and the time for scanning. The temperature of the water was also taken. Besides that, sweeping option in the PDA14 menu was enabled. In that menu as well, the region that signal transverses across was selected. Moreover, a filename was chosen and saved in the desired directory. The sample was then scanned. After the scanning was completed, the transducer was lifted up and the glass was taken out. The bone sample was then put under transducer. Step 8 was then repeated. However, this time the beam focus was made sure to be in the sample in a region of interest. “2D” option in the main menu was opened. A filename was chosen and saved in the desired directory. The settings for first and second axis depended on the desired illustration of the geometry, time of scanning, and quality of the image. If c-scan were to be done, the transducer would move along the first axis and would increase towards the direction of second axis. Shorter step size had be used if the loss of spatial information was to be avoided. However, this comes at the cost of scanning time. The formula to approximate scanning time is as follows:

\[
T_{\text{scanning}} = \frac{D_{1^{\text{st}}\text{ axis}}}{\text{Step}_{1^{\text{st}}\text{ axis}}} \cdot \frac{D_{2^{\text{nd}}\text{ axis}}}{\text{Step}_{2^{\text{nd}}\text{ axis}}} \left( T_{\text{sweep}} + T_{\text{movement}} \right)
\]

where \( T_{\text{sweep}} \) is the time taken for sweeping, \( T_{\text{movement}} \) is the time taken to move the transducer a step size, \( D_{1^{\text{st}}\text{ axis}} \) and \( D_{2^{\text{nd}}\text{ axis}} \) are the total distances travelled by transducer in 1\text{st} axis and 2\text{nd} axis, respectively, and \( \text{Step}_{1^{\text{st}}\text{ axis}} \) and \( \text{Step}_{2^{\text{nd}}\text{ axis}} \) are the distances chosen for transducer to move in each
time step. Normally a larger step size and smaller sweeping were chosen for initial scan to get the region of interest. After that, the scanning could proceed to a smaller step size and larger sweeping. Care has to be taken here so that the beam profile does not overlap too much. The formula to approximate the diameter of the beam profile was as below [8]:

$$W_F = \frac{2\lambda z}{d}$$

where $\lambda$ is the wavelength of the sound wave, $z$ is the distance from transducer’s face to focal zone, $d$ is the diameter of the transducer, SOS is the speed of sound of specific material while $f$ is the frequency of the transducer. The SOS of water, for instance, was about 1480 m/s. After the scanning was completed, Matlab was opened to view the image. A specially written file named Bmodeslice was placed in the same directory as the output file. In Matlab, Bmodeslice was typed. When prompted, the filename and the number of slice were entered. Then the data file would be processed to generate a slide view of the ultrasound scanning.

3.3.3 Analysis Procedure

A MATLAB® program called estimator_v3beta4 written by Professor William D. O'Brien’s group was used in order to determine the properties of region of interest (ROI). This estimator program could estimate the BSC from a set of radio frequency (rf) data. To estimate the BSC, backscatter measurements must be compensated for the effects of compensation throughout the scattering volume [9]. In this program, frequency-dependent attenuation compensation factor presented by O’Donnel and Miller [10] was used:

$$F[\alpha(f), x_0, z] = e^{4\alpha(f)x_0} \frac{4\alpha(f)z}{e^{2\alpha(f)}z} - e^{-2\alpha(f)z}$$

where $f$ is the frequency, $\alpha$ is the attenuation coefficient, $x_0$ is the distance from the transducer to the center of the scattering volume, and $z$ is the length of the scattering volume. Besides, in a variety of media over a finite bandwidth, the attenuation coefficient of ultrasonic waves appears to be adequately modeled by a power law dependence on frequency [11]. Therefore in this study, the attenuation was assumed to follow the power law $\beta f^n$, where $\beta$ is the estimated attenuation coefficient and $n$ is the estimated frequency power law exponent. Another important aspect in using this program is that a form factor has to chosen. The quantitative ultrasound (QUS) estimates depend on the theoretical model to describe the ultrasound scattering, where the
function that describes the scattering structure is called form factor (FF) [12]. In this study, a conventional FF, Gaussian FF, is used. In order to use this program, the ROI were determined from the Bmodeslice beforehand. Based on the past results, cartilage is found to form around the end of bone near the gap in dumbbell shape, for a treated scaffold case. The cartilage will then serve as template for bony islands which then link to the end of bone. It is believed that those bony islands will try to bridge the gap. Based on these considerations, the method to define ROI was described in Appendix E. One issue in analyzing these images is that we are not clear whether the scaffold still remains in the tissue. The scaffolds, made of polymer (HDDA), are mostly found to be extruded out of the body of the frog in the past results. The scaffold is 1.2 mm in diameter, with a hollow cylindrical hole of 0.8 mm diameter at its center. The pore leading to the hollow cylinder is square in shape with length of 0.12 mm. The scaffold image is shown in Fig. 3.3. Another result from the critical size study is that, beyond 4 to 5 mm (the critical size), the gap will not be able to regenerate from the cartilage. In this case, scar tissue (or fibrous tissue) will form in the gap.

After the ROI had been defined, the slice number for analysis was chosen from Bmodeslice and the settings were saved to a file called settings.mat. The parameters for this program are shown in Table 3.1. Finally, the program estimator_v3beta4 was run.

3.4 RESULTS

The B-mode slices from ultrasound scanning are included in Appendix D. Appendix E shows the frog sent for analysis. Note that only five frogs were sent for analysis this time since scanning consumes plenty of time. Besides, sometimes gap could not be detected since the samples were cut at joints and they were round in shape. Therefore the place where the surgery was performed could not be obtained easily (Later I was told by my colleague from Institute for Genomic Biology who performed the surgeries that there was light scar on the skin where the surgery was performed). Appendix F shows the plots from the analysis of the regions in Appendix E. Note that the first five plots were to test the hypothesis whether the regions postulated were the same as predicted. Therefore BSC data of the similar postulated tissues, or the regions which had predicted similar properties, were grouped together. The other five plots grouped the BSC data of all the regions of the same sample into one plot. In other words, the first 5 charts will define the range of BSC values for a specific tissue while the following 5 charts will provide a view on
BSC values for all regions of a sample. Several observations could be made based on these plots. For the observation below, the maximum and minimum values of BSC for each region in the range of 15 MHz to 25 MHz were obtained. Since cartilage serves as template for ossification, we might expect its BSC values to be lower than that of bone but higher than that of soft tissue.

1. From chart 1, the trends for region 1, 2, 3, 4, and 14 are almost the same, except frog11_region 2 and frog6, region2. They are in the range of 1e-3 to 1e-1 dB/cm, with most concentrated at 4e-3 to 4e-2 dB/cm.

2. From chart 2, BSC of regions 6 are generally higher than that of region 5. However, since we don’t know which direction is proximal or distal (as the sample is in cylindrical shape when I obtained it), we could not conclude what it implies here. Regions 6 are in the range of 1e-3 to 1e-2 dB/cm. Regions 5 are in the range of 4e-5 to 4e-4 dB/cm.

3. From chart 3, the region7, which is at the center of the gap, is found in the range of 1e-4 to 2e-3 dB/cm.

4. From chart 4, regions 8 and regions 9 are found in the range of 2e-5 to 7e-4 dB/cm, except frog-11 region 8 which data is taken at tissue with high reflection value.

5. From chart 5, most regions 10, 11, 12, 13 have values in the range of 3e-5 to 1e-3 dB/cm, except frog11_region, and frog6_13.

6. For chart 6 (frog 11), regions 2, 3, 4, 14 are different from region 5, 7, 8, 9, 11, 13. Region 6 falls in bone region, suggesting region 6 in this sample might have ossified tissue (or possibly has cartilage), while region 12 falls in soft tissue region.

7. For chart 7 (frog 2), regions 1, 2, 4 are different from 5, 8, 9, 10, 11. Regions 3, 6, 7 are between them. Region 6 should have ossified tissue (or possibly has cartilage). Though trend of region 3 appears to be decreasing with increasing frequency, it does fall into bone region. Region 7 falls into soft tissue region.

8. For chart 8 (frog 5), regions 1, 2, 3, 4 are distinctly different from regions 8, 9, 11. Regions 7, 10, 12, and 13 are in between the latter two groups of regions. Regions 12 should have ossified tissue (or possibly has cartilage) since it is centered on 1e-3 dB/cm. No conclusion could be made on region 7, 10, and 13 since they have BSC curves much higher than the soft tissue region and extend into the soft tissue region.
9. For chart 9 (frog 14), regions 3, 4 are different from 9, 10, 11. Regions 6 and 14 are between them and they should be ossified tissue (or possibly having cartilage). No conclusion can be made on regions 7, 12, 13.

10. For chart 10 (frog 6), regions 2, 4, 14 are different from regions 5, 7, 11, 12. Regions 3, 6 are between them. Region 9 is the lowest and falls into soft tissue region category. Region 13 extends from the bone region to the soft tissue region. Region 3 falls in bone or cartilage region while region 6 possibly has cartilage.

3.5 DISCUSSION
Based on the above observation, the regions which contain bone (region 1, 2, 3, 4, and 14) can be clearly separated from other regions. The bone regions generally have higher BSC values than other regions. This phenomenon is to be expected since bone could reflect more wave back than other soft tissue as it has higher reflection coefficient compared with the soft tissue around it. Region 8 and region 9 are confirmed in the soft tissue regions which do not include cartilage since these regions typically have lower BSC values compared with other regions. Besides, the hypothesized cartilage regions (region 5, 6, 7, 10, 11, 12, and 13) are difficult to be confirmed. Some of those regions could be said to contain cartilage since it has distinctively higher BSC value than the soft tissue region, such as region 6 in frog11, region 6 in frog2, region 12 in frog 5 and others as specified in the result section. While others such as region 12 in frog5, region 7 in frog2 and others could be determined to be in soft tissue region based on the much lower BSC values. Other regions besides those mentioned could not be decided as they have values in between cartilage and soft tissue region, and sometimes the curves will cross between these two regions.

More data will need to be collected to have a convincing and distinctive region of cartilage in order to differentiate it from bone region and soft tissue region. On the other hand, this study shows the feasibility of using ultrasound to measure bone growth non-invasively. By measuring the length of the gap at a certain time interval, the bone growth rate can be determined. This can provide valuable input to model bone growth mathematically and computationally.

3.6 CONCLUSIONS
The feasibility of applying ultrasound technique to detect cartilage tissues and calcified tissues was applied in this study. The result showed that ultrasound holds promising potential to identify
bone region and therefore it can be applied to capture growth of bone tissue in-vivo. This can provide valuable input to model bone growth mathematically and computationally. As for cartilage tissue, more data will need to be taken in order to obtain a distinctive BSC signature which will differentiate the cartilage region from other soft tissues.

3.7 REFERENCES


3.8 FIGURES AND TABLES

Table 3.1 Input parameters when using program estimator_v3beta4. \textit{gain} is the gain between the reference and the sample, \textit{flo} is the lower frequency analysis bandwidth, \textit{fhi} is the upper frequency analysis bandwidth.

<table>
<thead>
<tr>
<th>$\beta$</th>
<th>$n$</th>
<th>Reflection coefficient</th>
<th>Transducer frequency</th>
<th>\textit{gain}</th>
<th>\textit{flo}</th>
<th>\textit{fhi}</th>
<th>Form factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>Plexiglass</td>
<td>20 MHz</td>
<td>0</td>
<td>10 MHz</td>
<td>30 MHz</td>
<td>Gaussian sphere</td>
</tr>
</tbody>
</table>

Figure 3.1 Adult \textit{Xenopus laevis} as a model system for bone regeneration: Tarsus bone extirpation. a) Schematic drawing showing the bone structure of the \textit{Xenopus} hind limb, indicating the area of the tarsus removed for our studies. b) Photo of adult frog taken 6 wk after surgery to remove part of the tarsus bone. The arrow indicates the surgically altered left tarsus segment of the limb, which has healed properly after the procedure. c) Micro CT scan image of the tarsus segments from the frog in a), showing the gap produced by the removal of bone tissue. The intact right tarsus is shown on the right side of the panel.
Figure 3.2 Ultrasonic scanning equipment in Bioacoustic Research Laboratory
a) Daedal micro-positioning system which allows movement along three translational axes and around two angular axes, b) ultrasonic scanning with transducer above the sample in the water tank.

Figure 3.3 High resolution 3D microfabrication of model frog limb scaffold.
CHAPTER 4

INVESTIGATION OF USING OPTICAL COHERENCE TOMOGRAPHY SCANNING ON BONE

4.1 INTRODUCTION
Optical coherence tomography (OCT) is an imaging technique that permits the imaging of tissue microstructure in situ non-invasively [1]. It is comparable to ultrasound B mode imaging except that it uses low coherence light rather than sound and performs cross-sectional imaging by measuring the backscattered intensity of light from structures in tissue. It generally has one- to two-order-of-magnitude higher spatial resolution than ultrasound since it uses light. Originally it was used to image an eye since eye is transparent and optically accessible. Recently OCT has been applied for imaging in a wide range of nontransparent tissues other than eyes. However, the imaging depth is limited by optical attenuation due to scattering and absorption. Nonetheless, imaging depth of 2-3mm is still achievable. In this study, the possibility of applying OCT technique on bone growth of a surgically tarsus removed frog limb, as in the ultrasound section, is investigated.

4.2 METHODOLOGY
Two samples of frog hind limb were scanned using OCT equipment with the help of personnel from Professor Boppart’s group in Carle Hospital. Part of sample 1 has been removed of skin and soft tissue to expose bone while part of sample 2 has been removed of skin but soft tissue remained intact. The samples were then scanned at different locations.

4.3 RESULTS AND DISCUSSION
Figure 4.1 to 4.4 shows that OCT could be used to detect the microstructure of frog hind leg non-invasively. Striation of muscle could be detected and the bone region is detected as a dark region which appears after it, which is as shown in Fig. 4.1 and Fig. 4.4. While from Fig 4.2, the bone region could be detected due to a clearly visible layer in between the skin and the bone. However, the drawback of this technique is that it has a limited depth penetration if the tissue is not light permissible. 2-3mm of depth is the limit of this technique and everything after this limit will appear as dark regions. Though the frog tarsus is located approximately at 2mm below the skin
surface, its presence could not be confirmed as shown in Fig 4.3 since the dark region appears on
the same level as the other region where the signal has been attenuated. In addition, there is
difficulty in detecting bony islands in the gap using this technique since those regions are
generally smaller than the tarsus and their existence could not be confirmed based on black spots
on the diagram.

Ultrasound, on the contrary, has a better depth penetration and it can detect the bone
pieces and bony islands easily. Though the resolution of ultrasound is much lower than that of
OCT, 200-300 µm of resolution is sufficient for our goal to detect the bone growth. These are the
reasons ultrasound is used in this study instead of OCT.

4.4 CONCLUSIONS
The feasibility of applying optical coherence tomography (OCT) to detect bone growth in frog
limb was performed. Due to insufficient depth penetration, the existence of bone cannot be
confirmed through this technique.

4.5 REFERENCES
   Tomography Imaging in Developmental Biology. *Methods in Molecular Biology, Vol. 135:
   Developmental Biology Protocols, Vol. I* Edited by: R. S. Tuan and C. W. Lo @ Humana
Figure 4.1 OCT scanning of sample 1 across the joint of frog hind limb. Striations of muscle fibers are clearly visible. Besides, the darker region appears at scanning across the joint since bone does not allow light to pass through it.
Figure 4.2 OCT scanning across the joint of sample 1.

Figure 4.3 The region of bone could not be determined based on this diagram.
Sample 2

- Thin layer of connective tissue
- Skin
- Stratum corneum of skin
- Muscle (No striation since we scan across it)
- Bone region

Figure 4.4 OCT scanning across the joint of sample 2.
APPENDIX A

PROBABILITY FUNCTIONS FOR DATA FITTING

Beta

\[ f(x; a_1, a_2, \delta_1, \delta_2) = \frac{\left( \frac{x - \delta_1}{\delta_2 - \delta_1} \right)^{a_1-1} \left( 1 - \frac{x - \delta_1}{\delta_2 - \delta_1} \right)^{a_2-1}}{(\delta_2 - \delta_1) B(a_1, a_2)} \]  \hspace{1cm} (A.1)

Chi

\[ f(x; \alpha, \beta, \varepsilon) = \frac{2^{1-\alpha} \left( \frac{x - \varepsilon}{\beta} \right)^{\alpha-1} e^{-\frac{1}{2} \left( \frac{x - \varepsilon}{\beta} \right)^2}}{\beta^{\alpha/2} \Gamma\left( \frac{\alpha}{2} \right)} \]  \hspace{1cm} (A.2)

Gamma

\[ f(x; \kappa, \lambda, \varepsilon) = \frac{\lambda^\kappa [\lambda(x - \varepsilon)]^{\kappa-1} e^{-\lambda(x - \varepsilon)}}{\Gamma(\kappa)} \]  \hspace{1cm} (A.3)

Gumbel Max

\[ f(x; \mu, \alpha) = \alpha e^{(\alpha(x - \mu) - e^{\alpha(x - \mu)}} \]  \hspace{1cm} (A.4)

Gumbel Min

\[ f(x; \mu, \alpha) = \alpha e^{(\alpha(x - \mu) - e^{\alpha(x - \mu)}} \]  \hspace{1cm} (A.5)

Logistic

\[ f(x; a_1, a_2) = \frac{1}{4a_2 \cosh^2 \left( \frac{x - a_1}{2a_2} \right)} \]  \hspace{1cm} (A.6)

Normal

\[ f(x; \mu, \sigma) = \frac{1}{\sqrt{2\pi}\sigma} e^{-\frac{1}{2} \left( \frac{x - \mu}{\sigma} \right)^2} \]  \hspace{1cm} (A.7)

Rayleigh
\[ f(x; \alpha, \varepsilon) = \frac{x - \varepsilon}{\alpha^2} e^{-\frac{1}{2} \left( \frac{x - \varepsilon}{\alpha} \right)^2} \]  
(A.8)

Rectangular

\[ f(x; a_t, \varepsilon) = \frac{1}{a_t} \]  
(A.9)

Weibull

\[ f(x; \mu, \kappa, \varepsilon) = \frac{\left( \frac{x - \varepsilon}{\mu - \varepsilon} \right)^{\kappa - 1}}{(\mu - \varepsilon)^\kappa} e^{\frac{x - \varepsilon}{\mu - \varepsilon}} \]  
(A.10)
APPENDIX B
INTERSECTED VOLUME CALCULATION

Figure B.1 (a) and (b) show the connection of two fibers at acute angle and obtuse angle, respectively.

Figure B.1 Calculating intersected volume at two fibers connection with (a) acute angle, (b) obtuse angle.

The intersected volume is calculated in the following manner. Vectors for fiber 1 and fiber 2, denoted by \( l_1 \) and \( l_2 \) respectively, are found through the equation B1.

\[
v_i = \frac{<x_i^b - x_i^a, y_i^b - y_i^a, z_i^b - z_i^a>}{\sqrt{(x_i^b - x_i^a)^2 + (y_i^b - y_i^a)^2 + (z_i^b - z_i^a)^2}}
\]  
(B.1)

where \( x, y, z \) are coordinates of the nodes, superscripts \( a \) and \( b \) are two end nodes of the element, and subscript \( i \) denotes fibers connecting at the joint, with value 1 or 2.

The angle at the joint can then be found through equation B2.

\[
\theta = \cos^{-1}(v_1 \cdot v_2)
\]  
(B.2)

Through geometry of the triangle, angle \( \theta \) can be divided into angles \( \alpha \) and \( \gamma \), as shown in Fig. B.1 (a). The intersected volume for obtuse angle, which consisted of two triangles \( V_1 \) and \( V_2 \), is then calculated as below.

\[
\alpha = \theta - \frac{\pi}{2}
\]  
(B.3)

\[
\gamma = \frac{\theta - 2\alpha}{2}
\]  
(B.4)

\[
l_1 = r \tan \gamma
\]  
(B.5)

\[
V_{tot} = V_1 + V_2 = \frac{1}{2}l_1\pi r^2
\]  
(B.6)
where \( r \) is the radius of the fiber, and angles \( \alpha, \theta \) and \( \gamma \) are denoted in radians.

The same principle can be applied to acute angle, with little modifications.

\[
\alpha = \frac{\pi}{2} - \theta \quad \text{(B.7)}
\]

\[
\gamma = \frac{\theta}{2} \quad \text{(B.8)}
\]

\[
l_2 = \frac{r}{\cos \alpha} \quad \text{(B.9)}
\]

\[
V_{\text{tot}} = \pi r^2 l_2 - \frac{1}{2} l_1 \pi r^2 \quad \text{(B.10)}
\]

It may appear that the calculation of intersecting volume is easy to carry out. However, when it is implemented throughout the network, several issues arise. One of the issues is that when two fibers are connected at obtuse angle, there is a small gap on the opposite side of the intersected volume, as shown in Fig. B.1 (b). If these two fibers are viewed as a whole piece as shown in Fig. B.2 (a), then this piece will have a chip which serves as a stress concentration side. This will weaken the fiber connection and thus the whole network, resulting in remarkably lower strength value. Since nature always selects the best way to accommodate the environment, and TEM image in the previous section shows no irregularities or chips along the fiber, therefore the connection at obtuse angle is modeled to have no gap, as shown in Fig. B.2 (b).

![Figure B.2 Obtuse angle connection (a) With gap, (b) No gap.](image)

The FORTRAN program establishes the connection by linking two fibers which have intersected volume and makes a common node to those two fibers. This action results in four elements and five nodes from initial two fibers. Therefore for every joint there will be at least two elements which are almost parallel to each other. Intersected volume calculation will not be performed on these two elements, but will be performed on the following third and fourth elements that are almost parallel to each other. MATLAB® script is written to look for the nodes
serving as the joints, identify all elements at the joints, find the two parallel elements and make them the master elements at a particular joint and the other two as slave elements. Calculations based on equation B1 through B10 are then performed on the slave elements. This operation is finally extended to the whole network.

The other issue arising in calculating the intersected volume is that the calculations above assume that fibers have lengths much longer than $l_2$. The figures and formulations below detail conditions where fibers have lengths which are around and below $l_2$.

Generally, the intersected volume can be calculated through the following equation

$$V_{tot} = \pi r^2 l_2 - \frac{1}{2} l_1 \pi r^2 - \pi r^2 l_f$$

(B.11)

where $l_f$ is length factor which is different according to the condition of intersecting as in figure 11. Generally, the $l_f$ term is calculated by taking the ratio of the intersected area over the area viewed from one side for the length covering the intersected area, multiplied by the volume of that specific length.

The condition in Fig. B.3 (a) can be denoted by the following inequalities

$$l_2 \leq l_{f1} < l_2 (1 + \sin \alpha) ; l_{f2} > l_{f1} \sin \alpha + r \cos \alpha$$

where $l_{f1}$ and $l_{f2}$ are lengths of fiber 1 and fiber 2, respectively.

The length factor for this case is

$$l_f = \left[ \frac{r}{\tan \theta} - \frac{(l_{f1} - l_2)}{4r} \right] \tan \theta$$

(B.12)
Figure B.3 (a) – (i). Different configurations of volume intersections.

The condition for Fig. B.3 (b) where the ends of the fibers are intersecting can be described by the inequalities below.

\[ l_2 < l_{f1} < l_2(1 + \sin \alpha) \quad ; \quad r \sin \theta < l_{f2} < l_{f1} \sin \alpha + r \cos \alpha \quad \text{if} \quad l_2 \sin \alpha + \frac{(l_{f1} - l_2)}{\cos \theta} < r \sin \theta \quad \text{or} \]

\[ l_2 \sin \alpha + \frac{(l_{f1} - l_2)}{\cos \theta} \leq l_{f2} < l_{f1} \sin \alpha + r \cos \alpha \quad \text{if} \quad l_2 \sin \alpha + \frac{(l_{f1} - l_2)}{\cos \theta} > r \sin \theta \]

The length factor is

\[
lf = \frac{1}{2} \left[ \frac{r}{\tan \theta} - \frac{(l_{f1} - l_2)^2}{\tan \theta} \right]^2 \tan \theta + \frac{(l_{f1} \sin \alpha + r \cos \alpha - l_{f2})^2}{2 \sin \theta \cos \theta}
\]

\[
2r
\]  

(B.13)

For the third condition in Fig. B.3 (c), the inequalities are

\[ l_2 < l_{f1} < l_2(1 + \sin \alpha) ; \quad l_2 \sin \alpha + \frac{(l_{f1} - l_2)}{\cos \theta} \leq l_{f2} < r \sin \theta \]
The length factor is

\[
lf = \frac{1}{2} \frac{1}{\tan \theta} \left[ \frac{r}{\tan \theta} - (l_{f_1} - l_2) \right]^2 \tan \theta + \frac{2r \cos \theta - l_{f_2}}{\tan \theta} + \frac{l_{f_1} \tan \alpha}{4r} l_{f_1} \tag{B.14}
\]

For the fourth condition in Fig. B.3 (d), the inequalities are

\[
r \cos \alpha \leq l_{f_1} < l_2; \quad l_{f_2} > l_{f_1} \sin \alpha + r \cos \alpha
\]

The length factor is

\[
lf = \frac{1}{2} \frac{1}{\tan \theta} \left[ \frac{r}{\tan \theta} - (l_{f_1} - l_2) \right] + \frac{r}{4 \tan \theta} \tag{B.15}
\]

In the fifth condition in Fig. B.3 (e), the ends of the fibers are intersecting, and the inequalities are as follows

\[
r \cos \alpha \leq l_{f_1} < l_2 \quad ; \quad r \sin \theta \leq l_{f_2} < l_{f_1} \sin \alpha + r \cos \alpha \quad \text{if} \quad l_2 \sin \alpha - \frac{l_2 - l_{f_1}}{\cos \theta} < r \sin \theta \quad \text{or}
\]

\[
\frac{l_2 \sin \alpha - l_{f_1}}{\cos \theta} \leq l_{f_2} < l_{f_1} \sin \alpha + r \cos \alpha \quad \text{if} \quad l_2 \sin \alpha - \frac{l_2 - l_{f_1}}{\cos \theta} > r \sin \theta
\]

and the length factor is

\[
lf = \frac{1}{2} \frac{1}{\tan \theta} \left[ \frac{r}{\tan \theta} - (l_{f_1} - l_2) \right] + \frac{(l_{f_1} \sin \alpha + r \cos \alpha - l_{f_2})^2}{4r \sin \theta \cos \theta} + \frac{r}{4 \tan \theta} \tag{B.16}
\]

For the sixth condition in Fig. B.3 (f), the inequalities are as follows:

\[
r \cos \alpha \leq l_{f_1} < l_2 \quad ; \quad l_2 \sin \alpha - \frac{l_2 - l_{f_1}}{\cos \theta} \leq l_{f_2} < r \sin \theta
\]

The length factor is

\[
lf = \frac{2r \cos \theta - l_{f_2}}{\tan \theta} + \frac{l_{f_1} \tan \alpha}{4r} l_{f_1} + \frac{r}{2 \tan \theta} \left( l_{f_2} - l_{f_1} \right) + \frac{r}{4 \tan \theta} \tag{B.17}
\]

In the seventh condition in Fig. B.3 (g), the inequalities are

\[
r \cos \alpha > l_{f_1} \quad ; \quad l_{f_2} > l_{f_1} \sin \alpha + r \cos \alpha
\]

The intersected volume for this case is
\[ V_{\text{tot}} = \frac{1}{2} l_{f1} \left[ 1 + \frac{l_{f1} \tan \alpha}{2r} \right] \pi r^2 \]  \hspace{1cm} (B.18)

For the eighth condition in figure 11 (h), the inequalities are

\[ r \cos \alpha > l_{f1}; \quad l_{f1} \sin \alpha + r \cos \alpha > l_{f2} \geq r \cos \alpha \]

The intersected volume for this case is

\[ V_{\text{tot}} = \left[ l_{f1} r + \frac{l_{f1}^2 \tan \alpha}{2} - \frac{(l_{f1} \sin \alpha + r \cos \alpha - l_{f2})^2}{2r} \right] \pi r^2 \]  \hspace{1cm} (B.19)

For the last case in Fig. B.3 (i), the inequalities are

\[ r \cos \alpha > l_{f1}; \quad l_{f2} < r \cos \alpha \]

The intersected volume is

\[ V_{\text{tot}} = \frac{r + \frac{l_{f1} \tan \alpha}{2} - \left( \frac{r \cos \theta - \frac{l_{f2}}{\tan \theta}}{\cos \theta} + \frac{l_{f1} \tan \alpha}{2} \right)}{2} l_{f1} \pi r \]  \hspace{1cm} (B.20)

The above nine equations are also repeated by interchanging \( l_{f1} \) and \( l_{f2} \) while other parameters remain the same. This process will take care of the following cases:

\[ l_2 \sin \alpha - \frac{l_2 - l_{f1}}{\cos \theta} > l_{f2} \geq r \sin \theta \]

\[ r \sin \theta > l_{f2} > 0 \]
Appendix C

MODELING OF OSTEONAL LAMELLA AND INTERSTITIAL LAMELLA USING A CLASSICAL LAMINATE THEORY

Here, we provide the formulation of the classical laminate theory for a linear elastic general anisotropic material. Assuming $x_3$ to be the through thickness direction, the general constitutive law can be expressed as

$$
\begin{pmatrix}
\sigma_{11} \\
\sigma_{22} \\
\sigma_{33} \\
\sigma_{23} \\
\sigma_{31} \\
\sigma_{12}
\end{pmatrix} =
\begin{pmatrix}
c_{11} & c_{12} & c_{13} & c_{14} & c_{15} & c_{16} \\
c_{21} & c_{22} & c_{23} & c_{24} & c_{25} & c_{26} \\
c_{31} & c_{32} & c_{33} & c_{34} & c_{35} & c_{36} \\
c_{41} & c_{42} & c_{43} & c_{44} & c_{45} & c_{46} \\
c_{51} & c_{52} & c_{53} & c_{54} & c_{55} & c_{56} \\
c_{61} & c_{62} & c_{63} & c_{64} & c_{65} & c_{66}
\end{pmatrix}
\begin{pmatrix}
\varepsilon_{11} \\
\varepsilon_{22} \\
\varepsilon_{33} \\
\varepsilon_{23} \\
\varepsilon_{31} \\
\varepsilon_{12}
\end{pmatrix},
$$

(C.1)

where $\sigma_{ij}$ are the components of the stress tensor, $\varepsilon_{ij}$ are the components of the strain tensor, and $C_{ij}$ are the components of the stiffness tensor. After some simplification through the above-mentioned assumptions, the constitutive law can be expressed as

$$
\begin{pmatrix}
\sigma_{11} \\
\sigma_{22} \\
\sigma_{12}
\end{pmatrix} =
\begin{pmatrix}
Q_{11} & Q_{12} & Q_{13} \\
Q_{21} & Q_{22} & Q_{23} \\
Q_{31} & Q_{32} & Q_{33}
\end{pmatrix}
\begin{pmatrix}
\varepsilon_{11} \\
\varepsilon_{22} \\
\varepsilon_{12}
\end{pmatrix},
$$

(C.2)

where the components of the abridged stiffness matrix for general anisotropic material, involving 21 elastic constants, are

$$
Q_{11} = c_{11} + c_{13} aa - \frac{c_{15}}{c_{35}} (c_{13} + c_{33} aa - c_{36} a - c_{36} caa e) - \frac{c_{16}}{e} (a + aac),
$$

$$
Q_{12} = c_{12} + c_{13} bb - \frac{c_{15}}{c_{35}} (c_{23} + c_{33} bb - c_{36} b - c_{36} cbb e) - \frac{c_{16}}{e} (b + bbc),
$$

$$
Q_{13} = c_{14} + c_{13} dd - \frac{c_{15}}{c_{35}} (c_{34} + c_{33} dd - c_{36} d - c_{36} cdd e) - \frac{c_{16}}{e} (d + ddc),
$$

$$
Q_{21} = c_{12} + c_{23} aa - \frac{c_{25}}{c_{35}} (c_{13} + c_{33} aa - c_{36} a - c_{36} caa e) - \frac{c_{26}}{e} (a + aac),
$$

$$
Q_{22} = c_{22} + c_{23} bb - \frac{c_{25}}{c_{35}} (c_{23} + c_{33} bb - c_{36} b - c_{36} cbb e) - \frac{c_{26}}{e} (b + bbc),
$$

$$
Q_{23} = c_{24} + c_{23} dd - \frac{c_{25}}{c_{35}} (c_{34} + c_{33} dd - c_{36} d - c_{36} cdd e) - \frac{c_{26}}{e} (d + ddc),
$$
\[ Q_{31} = c_{14} + c_{34}aa - \frac{c_{45}}{c_{35}}(c_{13} + c_{33}aa - c_{36} \frac{a}{e} - c_{36} \frac{aa}{e}) - \frac{c_{46}}{e}(a + aac), \]

\[ Q_{32} = c_{24} + c_{34}bb - \frac{c_{45}}{c_{35}}(c_{23} + c_{33}bb - c_{36} \frac{b}{e} - c_{36} \frac{bb}{e}) - \frac{c_{46}}{e}(b + bbc), \]

\[ Q_{33} = c_{44} + c_{34}dd - \frac{c_{45}}{c_{35}}(c_{34} + c_{33}dd - c_{36} \frac{d}{e} - c_{36} \frac{dd}{e}) - \frac{c_{46}}{e}(d + ddc), \]  \hspace{1cm} (C.3)

with

\[ aa = \frac{aj - ef}{eh - cj}, \]

\[ bb = \frac{bj - eg}{eh - cj}, \]

\[ dd = \frac{dj - ei}{eh - cj}. \]  \hspace{1cm} (C.4)

Parameters \( a, b, c, d, e, f, g, h, i, \) and \( j \) can be found by using the elastic constants of the ply’s material as follows

\[ a = c_{13}c_{55} - c_{15}c_{35}, \]

\[ b = c_{23}c_{55} - c_{25}c_{35}, \]

\[ c = c_{33}c_{55} - c_{35}c_{35}, \]

\[ d = c_{34}c_{55} - c_{45}c_{35}, \]

\[ e = c_{36}c_{55} - c_{56}c_{35}, \]

\[ f = c_{13}c_{56} - c_{16}c_{35}, \]

\[ g = c_{23}c_{56} - c_{16}c_{35}, \]

\[ h = c_{33}c_{56} - c_{36}c_{35}, \]

\[ i = c_{36}c_{56} - c_{66}c_{35}, \]

\[ j = c_{36}c_{56} - c_{66}c_{35}. \]  \hspace{1cm} (C.5)

Then, the strain values are set to unity in each turn to find the stress response for a particular ply. This process is repeated for the whole ply for different fibril orientations until the global stress response can be found. The matrix \( P \) representing the global response is then solved for their material properties.
\[
\begin{pmatrix}
\sigma_{11} \\
\sigma_{22} \\
\sigma_{12}
\end{pmatrix} = \begin{pmatrix}
P_{11} & P_{12} & P_{13} \\
P_{21} & P_{22} & P_{23} \\
P_{31} & P_{32} & P_{33}
\end{pmatrix}\begin{pmatrix}
e_{11} \\
e_{22} \\
e_{12}
\end{pmatrix},
\] (C.6)

with
\[
P_{ij} = \frac{1}{n} \sum_{i=1}^{n} Q_{ij}^e.
\] (C.7)

Since the response is of the in-plane isotropy, the material properties can be derived as
\[
E = \frac{p_{11}^2 - p_{12}^2}{p_{11}},
\]
\[
v = \frac{p_{12}}{p_{11}},
\]
\[
G = p_{33},
\] (C.8)

where \( E, v, G \) are, respectively, the elastic modulus, Poisson’s ratio, and the shear modulus of the whole laminate.

This formulation extends the results existing in literature to the more general fully anisotropic case.
APPENDIX D

ULTRASOUND SCANNING FIGURES OF THE FROG TARSUS

Some notes on ultrasound scanning

1. The nomenclature used for the files is as follows. The first 2 numbers represent the frequency used. The second number is the number of trial in the lab that day. After an underscore, it is followed by description of the sample. For example, 132_crosstarsus means 13 MHz transducer is being used, on second trial and it is a B-mode scan crossing the tarsus. If no frog number is specified, then the scanning is on frog normal hind limb. If a frog number is specified, then the scanning is on frog hind limb in which part of the tarsus has been removed. Some of them are replaced by scaffold treated with growth factor, untreated scaffold, or just an empty gap. Bone is normally 2-2.5 mm below the skin.

2. Since the sample is cylindrical in size, and it has been removed from the frog limb, we could not know where the bone with the gap is. We have to make a random educated cross tarsus scan to get a bone location, and then make a scan along tarsus to check whether it has gap (which is the location we want to scan).

3. For work on that day, I included the settings used at the line after date.

4. For every measurement, I included the step size used after the nomenclature. Sometimes I included the description of the scanning direction, or tilting the sample.

5. Beside every picture, I described the position where I took the measurement through schematic drawing. Most pictures have symbols as reference position from the previous scanning, to give a clear notion where the current scanning is. Below every scanning, I tried to conclude what I got from that picture. If it is left blank, then there is no conclusion or result could be made on that scanning.
Table D.1 Setting used for ultrasound scanning on Date: 9_18_2008.

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<td>26 dB</td>
<td>180°</td>
<td>16ksamples</td>
<td>3000 mV</td>
</tr>
</tbody>
</table>

Figure D.1 132_crosstarsusr (step size = 250).

Figure D.2 133_alongtarsus (step size = 250).
Figure D.3 134_crosstarsus2 (step size = 250).

Figure D.4 135_crosstarsus3_moreprecise (step size = 250).
Figure D.5 136_alongtarsus2 (step size = 250, step size = 250).

Figure D.6 137_crosstarsus_tarsusremoved (step size = 250).
Figure D.7 138_alongtarsus_tarsusremoved (step size = 250, step size = 250).

Figure D.8 139_crosstarsus_findbone (step size = 250, step size = 500).
Figure D.8 (cont.)

Figure D.9 1310_alongtarsus_afterfindbone (step size = 250).
Figure D.10 1311_alongtarsus_afterfindbone2 (0.25mm to the left of 1310) (step size = 250).

Figure D.11 1312_alongtarsus_afterfindbone3 (0.5mm to the right of 1311) (step size = 250).
Date: 9_26_2008

Table D.2 Setting used for ultrasound scanning on Date: 9_26_2008.

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<td>26 dB</td>
<td>180°</td>
<td>16ksamples</td>
<td>3000 mV</td>
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</tbody>
</table>

Figure D.12 203_crosstarsus2 (step size = 150). I suspect that I found a region of bone/gap.

Figure D.13 204_crosstarsus3 (step size = 150). Found bone tissue.
Figure D.14 205_alongtarsus (step size = 150). There was only 1 red dot. The sample was suspected not parallel to the direction of transducer.

Figure D.15 206_crosstarsus4 (reorient sample) (step size = 150). Some scar tissue might be found.
Table D.3 Setting used for ultrasound scanning on Date: 9_29_2008.

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<td>16ksamples</td>
<td>3000 mV</td>
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</table>

Figure D.16 202_frog2_crosstarsus (step size = 100). Found bone at 4mm.

Figure D.17 203_frog2_alongtarsus (step size = 100). Found a complete piece of bone. Therefore have to turn around the sample to get the other bone.
Figure D.18 204_frog2_crosstarsus (turn the sample 180 degrees) (step size = 100). Region of interest (ROI) was at 3.75mm or at 5mm?

Figure D.19 205_frog2_alongtarsus (step size = 100). The red line was too near to the surface. It should be connective tissue instead of bone.

Figure D.20 206_frog2_alongtarsus2 (step size = 100). Found a gap in bone.
Figure D.21 207_frog2_cscan (step size = 200, step size = 200). Gap confirmed on slide2.
Figure D.21 (cont.)
Figure D.22 208_frog2_cscancrosstarsus (step size = 200, step size = 200). I would like to see whether there was a gap at 8.75 mm. I would focus on slide 5 on next scanning.
Figure D.23 209_frog2_bzscancrosstarsus (c-scan in z-direction) (step size = 200, step size = 200). No result.
Figure D.24 2010_frog2_bzscancrosstarsus_inversedirection (c-scan in –ve z-direction) (step size = 200, step size = 200). No result.
Figure D.25 2011_frog2_bzscancrosstarsus_inversedirection2 (Move transducer down by 1mm) (c-scan in –ve z-direction) (step size = 200, step size = 200).
Figure D.26 2012_frog2_hzscancrosstarsus_inversedirection3 (Move transducer down by 1mm again) (c-scan in –ve z-direction) (step size = 200, step size = 200).
Figure D.26 (cont.).

Figure D.27 2013_frog2_alongtarsus (step size = 200). The gap has been identified. However, the tissue from the repair process is yet to be confirmed.
Date 10_1_2009

Table D.4 Setting used for ultrasound scanning on Date: 10_1_2008.

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<td>3000 mV</td>
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Figure D.28 202_frog16_crosstarsus (step size = 200). Bone at 8mm?

Figure D.29 203_frog16_alongtarsus (step size = 200). Found long piece of bone, but not sure.
Figure D.30 204_frog16_alongtarsus2 (down 1.5 mm in z-direction) (step size = 200). There are two pieces of bone below it.

Figure D.31 205_frog16_crosstarsus (Turn around limb by 180 degrees) (step size = 200). Bone at 5.2 mm?

Figure D.32 206_frog16_alongtarsus (step size = 200). Bone could not be detected.
Figure D.33 207_frog16_crosstarsus2 (step size = 200).

Figure D.34 208_frog16_cscan (step size = 200, step size = 200). First slide was ok. Was it showing where the bones were?
Figure D.34 (cont.).
Figure D.34 (cont.).
Figure D.34 (cont.).

Figure D.35 209_frog16_alongtarsus (step size = 200). Bone could not be found.

Figure D.36 2010_frog16_crosstarsus (step size = 200)
Figure D.37 2011_frog16_alongtarsus (step size = 200)
Figure D.38 2012_frog16_cscan (step size = 200, step size = 200). ROI is not in focus.
Figure D.39 2013_frog16_alongtarsus (down in z-direction by 0.5 mm) (step size = 200).

Figure D.40 2014_frog16_alongtarsus2 (down in z-direction by 1 mm) (step size = 200). I would look at 5.8 mm in next scanning.
Figure D.41 2015_frog16_crosstarsus (step size = 200). No result. I would scan on this sample again next time.

Figure D.42 2016_frog5_crosstarsus (step size = 200). Bone could not be found.
Figure D.43 2017_frog5_crosstarsus3 (step size = 200). Bone still could not be found.

Figure D.44 2018_frog5_crosstarsus4 (step size = 200).
Figure D.45 2019_frog5_alongtarsus (step size = 200).

Figure D.46 2020_frog5_alongtarsus2 (Tilt the sample) (step size = 200).
Figure D.47 Time was up. I would continue next time.
Table D.5 Setting used for ultrasound scanning on Date: 10_5_2008.

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Figure D.48 202_frog5_crosstarsus (step size = 200). Bone could not be found.

Figure D.49 203_frog5_crosstarsus2 (step size = 200). Bone still could not be found.
Figure D.50 204_frog5_crosstarsus3 (step size = 200). Found bone at 2.5mm?

Figure D.51 205_frog5_alongtarsus (step size = 200). Found some discontinuous band of sample.
Figure D.52 206_frog5_alongtarsus2 (downward 0.5mm) (step size = 200).
Figure D.53 207_frog5_cscan (step size = 200, step size = 200). Slide 7 is good. Tilt the sample after this so that ROI has flat surface to transducer.
Figure D.53 (cont.).

Gap narrowing from 5mm to 3 mm
Figure D.53 (cont.).
Figure D.54 208_frog5_alongtarsus (Tilt sample) (step size = 200).

Figure D.55 209_frog5_alongtarsus2 (Tilt sample) (step size = 200). There was a narrowing of gap from 5mm to 3mm. However, I had to compare with histological slides for confirmation.
Figure D.56 2010_frog6_crosstarsus (step size = 200). Found bone at 2.25 mm.

Figure D.57 2011_frog6_alongtarsus (step size = 200). I could only detect a piece of bone. A huge empty area was at its left side. This might be due to attenuation as the soft tissue at the left side was quite thick.
Figure D.58 2012_frog6_bzscan (step size = 200, step size = 200). Slide 6 was ok. Note that at the RHS piece of bone, it was empty. After checking with sample and comparing with Matlab slides, the conclusion was that there should be one whole piece of bone (or maybe scaffold) until the end. We could not see it in Matlab slides because the tissue above it was thicker than the tissue above observable bone (in Matlab slides). Therefore the unobservable bone (or scaffold) just was attenuated.
Figure D.59 2013_frog16_crosstarsus (step size = 200). Found bone.

Figure D.60 2014_frog16_alongtarsus (step size = 200).
Figure D.61. 2015_frog16_alongtarsus2 (Tilt the sample) (step size = 200). Found gap? However, there were a lot of soft tissues in the gap. Therefore I was not sure where the end of the bone was.

Figure D.62 2016_frog16_alongtarsus3 (downward by 1mm) (step size = 200).
Figure D.63 2017_frog16_bzscan (downward by 0.15m) (step size = 200, step size = 300). Found nothing.
Figure D.63 (cont.).
Figure D.64 Found nothing.

Figure D.65 2019_frog16_bzscan (step size = 200, step size = 300). No conclusion could be made on this sample.
Date: 10_7_2008

Table D.6 Setting used for ultrasound scanning on Date: 10_7_2008.

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<td>16ksamples</td>
<td>3000 mV</td>
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</table>

Figure D.66 202_frog14_crosstarsus (step size = 200). Found bone at 5.5 mm.
Figure D.67 203_frog14_alongtarsus (downward by 3.5mm) (step size = 200). Bone could not be found. However, was there bone at 5mm?

Figure D.68 204_frog14_crosstarsus (step size = 200)
Figure D.69 205_frog14_crosstarsus (step size = 200). Bone at 3.4 mm?

Figure D.70 206_frog14_alongtarsus (step size = 200). Bone could not be found. However, there were 2 red pieces at each end. Both of them were about 2.5 mm down surface.
Figure D.71 207_frog14_alongtarsus2 (upward by 2 mm) (step size = 200)

Figure D.72 208_frog14_crosstarsus (step size = 200). No conclusion.
Date: 10_12_2008

Table D.7 Setting used for ultrasound scanning on Date: 10_12_2008.

<table>
<thead>
<tr>
<th>Mode</th>
<th>PRF</th>
<th>Energy</th>
<th>Damping</th>
<th>HP filter</th>
<th>LP filter</th>
</tr>
</thead>
<tbody>
<tr>
<td>P/E</td>
<td>2kHz</td>
<td>1 µJ</td>
<td>50Ω</td>
<td>1MHz</td>
<td>50 MHz</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Attenuation</th>
<th>Gain</th>
<th>RF output phase</th>
<th>Sampling Frequency</th>
<th>Trigger Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 dB</td>
<td>26 dB</td>
<td>180°</td>
<td>16ksamples</td>
<td>3000 mV</td>
</tr>
</tbody>
</table>

Figure D.73 203_frog14_crosstarsus (step size = 200). Bone could not be found.
Figure D.74 204_frog14_crosstarsus2 (step size = 200). Found bone?

Figure D.75 Found connective tissue but not bone. Not in focus?
Figure D.76 206_frog14_crosstarsus3 (step size = 200). Bone at 11 mm?

Figure D.77 Bone could not be found.
Figure D.78 208_frog16_crosstarsus (step size = 200)

Figure D.79 209_frog14_cscan (step size = 200, step size = 200). Found bone?
Figure D.79 (cont.).
Figure D.79 (cont.).

Figure D.80 2010_frog14_cscan_inverse (step size = 200, step size = 200 (inverse)). Slide 3 was good. Were bony islands on the right hand side of the slides?? With cartilage?? Not clear. I had to compare with histology study.
Figure D.80 (cont.).
Date: 10_19_2008

Table D.8 Setting used for ultrasound scanning on Date: 10_19_2008.

<table>
<thead>
<tr>
<th>Mode</th>
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<th>LP filter</th>
</tr>
</thead>
<tbody>
<tr>
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</tbody>
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<tr>
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<td>26 dB</td>
<td>180°</td>
<td>16ksamples</td>
<td>3000 mV</td>
</tr>
</tbody>
</table>

Figure D.81 202_frog11_crosstarsus (step size = 200). Found bone at 3 mm.

Figure D.82 203_frog11_alongtarsus (step size = 200). Found long bone. I will focus at vertical = 19 mm in next scanning.
Figure D.83 204_frog11_cscan (step size = 200, step size = 200). Found some bone transversely at slide 9?
Figure D.83 (cont.).
Figure D.83 (cont.).

Scaffold??
Figure D.83 (cont.).
Figure D.84 205_frog11_cscan2 (step size = 200, step size = 200).
Figure D.84 (cont.).
Figure D.85 206_frog11_cscan3 (step size = 200, step size = 200).
Figure D.85 (cont.).
APPENDIX E
CATEGORIZATION OF TISSUE REGIONS OF FROG TARSUS IN ULTRASOUND SCANNING

<table>
<thead>
<tr>
<th>Region 1</th>
<th>Region 2</th>
<th>Region 3</th>
<th>Region 4</th>
<th>Region 5</th>
<th>Region 6</th>
<th>Region 7</th>
<th>Region 8</th>
<th>Region 9</th>
<th>Region 10</th>
<th>Region 11</th>
<th>Region 12</th>
<th>Region 13</th>
<th>Region 14</th>
</tr>
</thead>
</table>

Region 1 is for bone region far away from the gap on the left hand side.
Region 2 is for bone region far away from the gap on the right hand side.
Region 3 is for bone (or ossified tissue from repair process) near the gap on the left hand side.
Region 4 is for bone (or ossified tissue from repair process) near the gap on the right hand side.
Region 5 is for soft tissue (most probably cartilage) around bone (or ossified tissue) near the gap on the right side of region 3.
Region 6 is for soft tissue (most probably cartilage) around bone (or ossified tissue) near the gap on the left side of region 4.
Region 7 is for soft tissue (might be cartilage if it is 3 months treated sample, or soft tissue if it is untreated or 8 weeks sample) at the center of the gap.
Region 8 is for region of soft tissue above the center of the gap.
Region 9 is for region of soft tissue below the center of the gap.
Region 10 is for soft tissue (most probably cartilage) around bone (or ossified tissue) near the gap on top of region 3.
Region 11 is for soft tissue (most probably cartilage) around bone (or ossified tissue) near the gap on the top of region 4.
Region 12 is for soft tissue (most probably cartilage) around bone (or ossified tissue) near the gap below region 3.
Region 13 is for soft tissue (most probably cartilage) around bone (or ossified tissue) near the gap below region 4.
Region 14 is for bony island in the gap.
Figure E.1 204_frog11_cscan -slide 9 (3 months, 5.5mm treated scaffold).
Figure E.2 2010_frog14_cscan_inverse – slide3 (3 months, 7.5mm treated scaffold).
Figure E.3 2012_frog6_bzscan - slide6 (critical size study (3 months), 8mm untreated, no scaffold)
Figure E.4 207_frog5_cscan – slide7 (6 weeks, 5mm treated scaffold).
Figure E.5 207_frog2_cscan–slide2 (6 weeks, 5mm treated scaffold). Region 7, 8, 11, 13, might be soft tissue. There is a little overlapping between region 5, 6, and 7.
Figure F.1 Plot grouping BSC data of potential bone regions.
Figure F.2 Plot grouping BSC data of potential cartilage regions.
Figure F.3 Plot grouping BSC data of potential cartilage and other soft tissue regions.
Figure F.4 Plot grouping BSC data of other soft tissue regions.
Figure F.5 Plot grouping BSC data of potential cartilage regions.
Figure F.6 Plot grouping BSC data of all regions scanned in frog 11.
Figure F.7 Plot grouping BSC data of all regions scanned in frog 2.
Figure F.8 Plot grouping BSC data of all regions scanned in frog 5.
Figure F.9 Plot grouping BSC data of all regions scanned in frog 14.
Figure F.10 Plot grouping BSC data of all regions scanned in frog 6.